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REPRODUCTIVE CYCLES AND SUPERFETATION IN PŒCILIÏD FISHES

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Introduction

Reproductive cycles in the pœciliïd fishes are of unusual interest for three reasons: (1) this family of fresh-water fishes arose in the tropics and for the most part has remained under tropical and sub-tropical conditions where the great seasonal variations in light and temperature found in temperate and arctic zones do not exist; (2) all the species in the family are ovoviviparous. The embryos have fairly large yolk sacs and are retained in the ovarian follicles after fertilization until birth, necessitating a respiratory exchange between parent and embryos. The ovary consequently serves the double function of providing the female gametes and of providing a site and the proper conditions for retaining growing embryos; (3) superfetation occurs in some of the genera. In some instances two broods of embryos of different ages are present in the ovaries and in the most extreme case there may be at one time as many as six small separate broods.

Pœciliïd fishes have been reared in aquaria for years and some of the general facts concerning size of broods, intervals between broods, correlation between size of brood and female parent, variation of brood size and intervals between broods at the different seasons of the year, are well known. Bits of reliable information concerning reproduction in a number of genera and species are found in most of the popular books on aquarium fishes. One species in particular, *Gambusia affinis,* has been introduced into new areas in the tropics as a mosquito eradicator and its life history and reproduction have been well studied. Such studies have given relatively little attention, however, to the developing ovocytes within the ovary and the relation between these groups of ovocytes and the broods.

In order to study reproductive cycles effectively it has been found necessary to know the stages of development of the embryos and of the

ovocytes within the ovary and to correlate this information with the records of the reproductive cycles. Furthermore, it is necessary to have as much of this information as possible from single females. Consequently, as far as possible, it has been the procedure to secure the brood-bearing record of an individual and then kill the individual at a definite period after the bearing of a brood in order to examine the embryos and developing ovocytes in the ovary. By following this practice throughout the year data have been secured upon the following relations: (1) the total number of broods produced within a year; (2) the relative time interval between broods; (3) the relative size of broods; (4) the exact time of fertilization of a group of eggs as related to the birth of a previous brood and as related to younger ovocytes; (5) the period of time over which fertilization takes place; (6) the variation in stage of development within a single brood; (7) whether or not fertilization occurs so as to form a new brood before the former brood is voided (superfetation); (8) the relation of the interval between broods and of the size of broods to environmental factors.

Any large collection of gravid females made in the field furnishes material for a study of the stages in the ovocytes as related to the age of the embryos being retained in the ovary. Superfetation if it exists can also be studied in this type of material.

In the present study the following species have been studied by the method of maintaining laboratory cultures and dating and dissecting the gravid females: *Gambusia affinis, Gambusia holbrookii, Gambusia panuco, Mollienisia sphenops, Mollienisia latipinna, Xiphophorus helleri, Xiphophorus strigatus, Pseudoxiphophorus bimaculatus, Platypœcilus maculatus, Platypœcilus variatus, Lebistes reticulatus, Limia vittata, Heterandria formosa, Micropœcilia picta, Micropœcilia parœ, Pœcilistes pleurospilus* and *Quintana atrizona.* The following have been studied by the use of large numbers of living specimens in the field: *Xiphophorus helleri, Xiphophorus strigatus* and *Pseudoxiphophorus bimaculatus,* at Cordoba, Mexico (March, 1932); *Pœciliopsis infans* at Octolan, Mexico (April, 1932); *Mollienisia sphenops* and *Gambusia panuco* near Monterey, Mexico; *Mollienisia latipinna* and *Gambusia affinis* in Louisiana. Another field study was made of *Brachyrhaphus episcopi* and *Mollienisia sphenops* at Barro Colorado Island in the Panama Canal Zone during the summer of 1936.

The writer is indebted to Dr. Stillman Wright for a large collection of *Pœcilia vivipara* from Parahyba, Brazil.

Reproductive Cycles

Various species of pœciliïds have been observed for years in aquaria and the observation has been made many times in some species that once a female attains sexual maturity she produces broods at regular intervals of about thirty days. All cyclical character seems to have disappeared in the reproduction of the male, the production of sperm being practically continuous after sexual maturity is attained. In other species it has been noted that the regular interval between broods is approximately fifteen days, while in still another species the young are born with less regularity, in smaller numbers and with less interval between the broods. Various theories have been advanced to account for the different lengths of cycles.

Some few observations have been made of brood production under natural conditions where it has been noted that the brood interval is much the same as that observed in the aquarium-reared specimens. A marked retardation of reproduction occurs during the winter months and it is much more marked under natural conditions than in aquarium-reared specimens.

It has been noted also that mature specimens obtained in the field and introduced into the laboratory do not produce broods with the regularity of laboratory-reared specimens. The broods of the wild specimens tend to be produced at longer and more irregular intervals even after the females have been adapted to laboratory conditions for periods of fifteen months. The young of these same females, reared from the first in the laboratory, produce broods with more regularity. The true and complete picture of the reproductive cycle as it occurs under natural conditions can only be obtained from material living under natural conditions and for this purpose collections of considerable size are being made at monthly intervals of *Gambusia affinis* at Key West, Florida, and of *Brachyrhaphis episcopi* in the Panama Canal Zone. A study of the ovocytes and the embryos in the gravid ovaries will make it possible to indicate the events in the reproductive cycle for all seasons.

Reproduction without Superfetation

In some species the birth of a brood is accomplished before any younger group of ova has matured sufficiently to be fertilized. Consequently only one brood will be found in the ovary at one time and the embryos will be in about the same stage of development. However, the extent to which younger cells have developed at the time the embryos are evacuated varies greatly and the following account will begin with those species in which the next younger group of cells is quite small and relatively undifferentiated.

Gambusia Type.—In *Gambusia*, females produce broods at about thirty-day intervals during late spring and summer. When eggs have been fertilized and during the time in which the embryos are developing, the cells of the next wave are small. They remain small and latent until embryos have been voided and then grow and differentiate rapidly until ready for fertilization. In an ovary containing a brood of very young embryos the oldest and largest cells are about .25 mm. in diameter. Approximately two weeks later, when the embryos are about to be born, the cells are still small, being only .3 mm. in diameter. Two or three days after the birth of the embryos these same cells have increased to a diameter of .7 to 1.5 mm., almost the maximal size attained at fertilization. Fertilization occurs over a period of about five days, beginning about seven days after the voiding of the last brood of embryos. Due to the length of the period of fertilization the embryos of a single brood may be relatively far apart in their developmental stages. In all other species examined the fertilization period is short and the embryos show less range in stages of development. *Gambusia affinis, G. holbrookii* and *G. panuco* are all very similar in the peculiarities described above.

A number of studies have been made upon the reproductive cycles of *Gambusia affinis* and *Gambusia holbrookii* (Barney and Anson, 1921*a*; Hildebrand, 1917; Kuntz, 1913; Seal, 1911; Seale, 1917). The work was concerned principally with the number of broods produced by wild specimens each year and the external factors which affect fecundity. As a result of these studies it was learned that the interval between broods in *Gambusia affinis* was about thirty days during the spring months and as long as eighty-five days during the winter if the specimens were kept in the laboratory and supplied with adequate food and warmth. It was also learned that under natural conditions several broods were produced during spring and summer but that reproduction ceased altogether during the winter. Barney and Anson correlated their study of the brood-producing record with the finding of embryos of different stages of development in the ovary and concluded: (1) that a single annual reproductive cycle occurs in *Gambusia* in its natural habitat; (2) that fertilization of all the eggs for an annual cycle occurred at the same time; (3) that the small cells in the ovary did not develop to maturity during the year, and (4) that after fertilization and partial development the embryos did not proceed at the same rate, a sufficient number to form one brood reaching the birth stage, being evacuated and being followed at approximately monthly intervals by other batches of embryos until all eggs that had been fertilized were voided.

Kuntz (1913), on the other hand, assumed that "A considerable number of ova reach maturity at the same time. These, being fertilized,

give rise to a brood of young. After the birth of this brood another lot of ova reach maturity and, being fertilized, give rise to a second brood. Thus, perhaps, all the ova required for the several broods which are born during a spring and summer may be present in the ovary at the beginning of the season."

The writer is inclined to agree with Kuntz that different groups of ova reach maturity at different times and that each group is fertilized separately, for two reasons: first, all the young are born at practically the same time and none remain in the ovary; second, within ten days after birth another lot of ova, approximately equal in number to the brood born previously, has developed and is ready for fertilization. It may be implied from the statement of Kuntz that he assumes *Gambusia* has an annual reproductive cycle in which all the ova for the annual cycle are elaborated at the same time. These interpretations concerning an annual cycle fail to take into account that the pœciliïds arose under tropical conditions and that they have short, often-repeated cycles each similar to one complete annual cycle in a fish of the temperate zone. Under the alternating conditions of the temperate zone these short cycles are repeated as often as environmental conditions permit within one favorable season and then become retarded or cease until favorable conditions are re-established.

The ovarian cycle in *Mollienisia latipinna* and *Mollienisia sphenops* resembles that of *Gambusia*. During the spring and summer broods are born approximately thirty days apart. Ova destined for a new brood reach a maximal diameter of about .7 mm. by the time the embryos of the earlier brood are ready to be voided—about twice the size of the cells in *Gambusia* at the same stage. However, they grow rapidly and reach a diameter of over 2 mm. by the time they are ready for fertilization. Apparently fertilization does not extend over so long a period in *Mollienisia* as in *Gambusia* and the embryos, just after fertilization, do not show the wide range in stage of development characteristic of *Gambusia*.

Pseudoxiphophorus bimaculatus has an ovarian cycle very similar to that of *Mollienisia latipinna* and *Mollienisia sphenops*. However, the interval between broods is longer, being thirty-five to forty days during May, June and July. In an ovary containing a brood ready for birth the next younger group of cells has reached a diameter of only .7 or .9 mm. in diameter. After the brood has been evacuated, however, the cells grow rapidly to a large size, about 2.4 mm., before fertilization.

Lebistes Type.—The rhythm of brood production in this type is very similar to that in the *Gambusia* type. During late spring and summer broods are produced approximately every thirty days. A rec-

ord of brood production by ten females of *Lebistes reticulatus* is shown in Table I. The data are taken from unpublished records of Dildine and the writer. It will be noted that the most regular production of broods and the shortest interval between broods occur in the late spring and early summer months. In late fall and winter months the interval between broods is nearly as long as in the late spring when broods are produced from twenty-two to thirty days apart. The specimens were kept under fairly constant conditions as regards food, temperature and salt and gas content of the water. No artificial lighting was employed, however, and the exposure of the specimens to light was therefore con-

TABLE I

Showing dates of broods produced by ten specimens of *Lebistes reticulatus*. Numbers under months indicate day on which brood is produced. Intervals between broods are expressed in days.

Specimen Number	January	Interval	February	Interval	March	Interval	April	Interval	May	Interval	June	Interval	July	Interval	August	Interval	September	Interval	October	Interval	November	Interval	December	Interval
1											27	25	22	27	18	35	22			46	6			
2											30	22	22	27	18	35	22			44	6			
3							5	29	4	31	4													
4							30	26	26															
5							21	39	29	22	20	26	16	31	16									
6							1	33	4	27	1	34	4											
7									22	28	19	23	13											
8	18																						9	40
9		60	28			39	8	28	6	27	2												20	
10					30			32	2															

trolled by the seasonal waxing and waning of daylight. The increase and decrease in brood production paralleled the increase and decrease in daylight. To what extent increased exposure to light may serve as a stimulating agent to reproduction, possibly through the medium of a gonad-stimulating hormone from the pituitary, remains to be worked out experimentally.

In *Xiphophorus* and *Platypœcilus* the brood-bearing record is very similar to that in *Lebistes* with a tendency to a slightly longer period between broods. The same increase in the interval between broods during the winter months has also been noted.

Conditions in the ovary during various phases of the reproductive cycle could be determined only by using the period in which a brood is

voided as a first stage and then obtaining regular stages a few days apart up to the time for the voiding of the next brood. Such a series was obtained during the height of the reproductive season. The findings in a number of cases are included here to indicate conditions within the ovary. (1) A specimen of *Lebistes* was sacrificed immediately after it had produced a brood of nine young. No young were found in the ovary and it was evident that all of the brood had been born during a three or four-hour period. In the ovary were found six ova approximately 1.4 mm. in diameter, orange-yellow in color and somewhat transparent. Four additional eggs similar in structure and color but varying in diameter from 0.8 to 1.0 mm. apparently belonged to this group. Presumably all would have been fertilized to form the next brood. Another group of eight cells represented a still younger set which would have been fertilized still later. These younger cells could be distinguished from the older group of cells by their smaller size and difference in structure. The younger group contained cells white in color, opaque and less than 0.5 mm. in diameter with more variation in size than in the older group of cells. It should be emphasized that there were definite gaps in size between the older and the younger group of cells and also between this younger group and a still younger mass of cells in various stages of development. (2) A second specimen was sacrificed and the ovary examined eight days after a brood had been born. Fertilization had recently taken place in a group of ova about equal in number to the brood born eight days before. Some of the fertilized eggs had reached an early blastula stage, some were in early segmentation stages and two had apparently not yet been fertilized. The eggs in the group as a whole varied in diameter from 1.2 to 2.0 mm. One of the larger eggs had not yet been fertilized while several of the smaller ones of the group had been fertilized. Therefore, size, except within the limits indicated, is not an index of the fertilizability of an egg. A second group of cells, about equal in number to the group undergoing fertilization, was opaque and slightly yellow in color. These cells varied in diameter from 0.4 to 0.7 mm. in diameter. (3) The ovary of a specimen killed twenty-one days after the birth of its brood of six contained a new brood of seven, the individuals of which were 5 mm. long. Seven transparent yellow eggs, varying in diameter from .6 to .9 mm., represented the next oldest group. Two were slightly opaque, indicating that they were slightly younger than the others. Fifteen cells, the largest of which was about .25 mm. and the smallest less than .1 mm. in diameter, were white and opaque or had transparent centers and opaque white layers at the periphery.

The development of cells in the ovary up to the time that they be-

come mature and ready for fertilization may be summarized as follows:

1. A large number of transparent cells less than 0.1 mm. in diameter are ready to be drawn upon for differentiation.

2. During the first month some of these cells begin to differentiate and develop a white opaque layer at the periphery. A number of the cells reach a diameter of about 0.5 mm. and become white and opaque throughout. At the same time an older group of eggs and also a developing brood is present in the ovary.

3. During the second month the oldest members of this group of developing cells grow rapidly, become orange-yellow and translucent and reach a diameter of from 0.8 to 1.4 mm. by the end of the month. The number of cells undergoing this development is approximately equal to the number of individuals in a brood.

4. During the third month these orange-yellow translucent eggs comprise the oldest group in the ovary, the embryos of the previous brood having been born. During the first ten days of the month they grow to a maximal size of 2 mm., some being as small as 1.4 mm. in diameter, and at the end of this ten-day period all are fertilized. Variations in the stages of the young embryos indicate that fertilization does not take place in all simultaneously. Fertilization of all members of the group is accomplished within two days. During the remaining twenty days of the month the embryos develop and at the conclusion of the month they are voided. In the meantime younger waves of cells are passing through stages of development already described.

A comparison between the *Lebistes* and the *Gambusia* types indicates: (1) that, at the time of the birth of a brood of embryos, the ovocytes of the next younger group is much larger and better differentiated in *Lebistes* than in *Gambusia;* (2) that the period of time elapsing between the birth of a group of embryos and the fertilization of the next group of ova is shorter in the *Lebistes* type than in the *Gambusia* type.

Data obtained in *Xiphophorus* and *Platypœcilus* were strikingly similar to those obtained in *Lebistes.* The adults of *Platypœcilus* and *Xiphophorus* are larger than those of *Lebistes* but ova of the same age are equal in size. However, in *Lebistes* fewer eggs come to maturity at one time and the broods contain fewer individuals. Other species which have cycles like that of *Lebistes* or intermediate between the *Lebistes* and the *Gambusia* type are *Pœcilia vivipara, Micropœcilia picta, Micropœcilia parœ, Brachyrhaphis episcopi* and *Limia vittata.* Others not yet studied will undoubtedly be added to the list.

Quintana atrizona Type.—In this type there is a very short interval between the birth of a brood and the fertilization of the ova of the fol-

lowing brood. In a female killed and examined twenty hours after the birth of a brood, the next group of cells in the ovary was found to be fully developed. These ova, approximately equal in number to the brood just born, were 1.8 to 2.2 mm. in diameter. Some had apparently been fertilized a few hours before. Of this group—nineteen in all—seven had reached an advanced segmentation stage, nine were in earlier segmentation stages and three had not yet been fertilized. It was apparent that fertilization had taken place in most of the ova immediately after the evacuation of the previous brood and that fertilization in the remaining three cells would have been complete within a few hours more. Differentiation and growth of the ova next in line for fertilization had reached a final stage by the time the embryos of the previous brood were born and the interval between the two events was reduced almost to the vanishing point.

TABLE II

Illustrating production of broods in *Pœcilistes pleurospilus*

Date	No. of young in brood	Interval in days between broods
November 20	2	
December 16	2	26
January 8	2	23
February 1	2	23
February 21	3	20
March 15	3	22
April 3	7	19
April 25	7	22
May 10	8	16
May 24	9	14
June 5	9	12

If the ova should develop still more rapidly and reach their final stage of differentiation and growth before the older brood of embryos was evacuated a condition would exist in which it would be possible for fertilization to occur before the older brood had been evacuated and for two broods to exist in the ovary at the same time. Such a condition of superfetation does occur in the next species of pœciliïds to be considered.

Reproductive Cycles Involving Superfœtation

Pœcilistes pleurospilus and Pœciliopsis infans.—Specimens of *Pœcilistes pleurospilus* were observed from November to May in order to secure records of the broods produced. Single specimens were then sacrificed at different intervals after the production of a brood to determine the state of development of embryos and ova within the ovary. The record of broods produced is shown in Table II. The record

shows that: (1) the interval between broods was longest in the winter months and that it was becoming shorter in the early spring months; (2) the broods were becoming larger as the spring advanced; (3) in general, also, the broods were smaller than those of pœciliïd fishes in which superfetation does not occur; (4) during the spring months the interval between broods is much shorter than in *Lebistes*.

Stoye (1935) finds the interval between broods in this species to be ten to twelve days. This is apparently the record of specimens reproducing in May or June.

A female of *Pœcilistes pleurospilus*, killed two days after the last brood of eight young had been born, contained two broods of unborn embryos in the ovary. The older brood consisted of nine embryos which were well developed but far from the stage in which they would be born. The eyes were well developed and pigmented, and pigment cells were present over the brain, down the mid-dorsal line and along the lateral line. All embryos were in approximately the same stage of development. The second brood contained nine very young embryos, with only a few somites developed. A group of twelve cells about .5 mm. in diameter, together with many smaller undifferentiated cells, were also present in the ovary. Another female, sacrificed twelve days after she had produced a brood, contained an ovary in which two broods were well separated in age and also a group of developing ova nearly 2 mm. in diameter.

An abundance of preserved material of *Pœciliopsis infans* was available and forty-two ovaries were dissected. In every ovary two broods of embryos were found, together with a group of cells approximately equal in number to the embryos in a brood, well in advance of the development of a mass of smaller undifferentiated cells. Embryos of a single brood varied somewhat more in their degree of development than was the case in *Pœcilistes pleurospilus* but the least advanced member of the older brood was always separated by a considerable gap from the most advanced member of the younger brood.

The condition found by Henn (1916) in *Pseudopœcilia fria* and *Diphyacantha chacoënsis* indicates that the ovarian cycle of these forms is probably like that of *Pœciliopsis infans* and *Pœcilistes pleurospilus*. Henn dissected five ovaries and found in each a brood of large embryos, a second brood of small embryos with about the same number of individuals as in the first group and a small number of developing ova.

In the first two of these species, at least, the sequence of events for development of ova and embryos would be as follows: (1) a group of ova grow and differentiate until they reach a diameter of about 2 mm. At this point an older brood of embryos, *A*, is at the point of being

born and a much younger brood, *B*, is also present in the ovary; (2) the ova are fertilized, forming brood *C*, and the older brood, *A*, is born at practically the same time; (3) broods *B* and *C* develop for a period of 10 to 22 days and during this time another group of younger ova develop to the maximal size; (4) brood *B* is born, brood *C* is retained in the ovary and the eggs of the new group are fertilized to form brood *D*.

Superfetation, as observed in the species mentioned above, is apparently caused by the earlier development of the younger ova, allowing earlier fertilization. A sequence might be arranged from the species described so far, illustrating regular stages through which superfetation might arise. (1) In *Gambusia* and *Mollienisia* the ova remain quite small until the birth of the brood already contained in the ovary; they may not reach a state in which they can be fertilized for about two weeks. (2) In *Lebistes* the developing ova are much larger and better developed when the brood in the ovary is born and this continued growth brings them to the point of fertilization a few days earlier. (3) In *Quintana atrizona* the ova have reached a still more advanced stage at the birth of the brood in the ovary; they have reached their maximal size and complete differentiation and fertilization takes place immediately. (4) The ova in *Pœciliopsis infans* and *Pœcilistes pleurospilus* attain their full size and complete differentiation long before the brood in the ovary is born. Fertilization of the ova takes place and the resulting embryos are retained in the ovary as a second brood.

Heterandria formosa.—This species has small broods with short intervals between the broods.

The records shown in Table III are those of two young females that have just attained sexual maturity. The most important features in the records are: (1) the broods are very small, one or two embryos being produced at a time during the winter months and as many as five or six at one time during May and June; (2) the interval between broods is long during the winter months, thirty-three to forty-one days. It becomes shorter with the approach of spring, reaching the shortest interval —three to eight days—in May and June. Seal (1911) made a record of broods produced by larger specimens during the months of July and August. He found that the broods containing from four to sixteen embryos were born at intervals of from four to nine days.

A female which had produced a brood of nine was sacrificed and examined on May thirteenth. The ovary was well filled with embryos and when these were dissected out of their follicles and arranged in groups it was found that six levels of development could be distinguished. There were nine embryos at the oldest stage (brood *A*) and these would presumably have been born about seven days later. All

external parts of the embryos were well developed except the caudal fin, and the yolk sac was almost completely absorbed. They were about 5 mm. in length (snout to base of caudal fin). Eight embryos formed

TABLE III

Brood production in *Heterandria formosa*

Specimen No. 1

Date	No. of young in brood	Interval in days between broods
December 12	1	
January 24	2	33
February 9	2	16
March 1	2	19
March 17	2	16
March 29	3	12
April 6	2	8
April 14	3	8
April 26	2	12
April 30	3	5
May 4	2	4
May 12	5	8
May 20	3	8
May 26	2	6
June 2	3	7
June 8	2	6
June 14	3	6
June 17	5	3
June 20	4	3
June 28	3	8
July 1	1	5
July 11	2	10
July 25	1	14

Specimen No. 2

Date	No. of young in brood	Interval in days between broods
December 10	2	
January 21	2	41
February 7	2	17
February 28	2	21
March 6	1	6
March 17	3	11
April 4	2	18
April 7	2	3
April 20	2	13
April 26	3	6
April 30	1	4
May 9	3	9
May 12	2	3
May 20	2	8
May 26	6	6
May 31	4	5

brood *B*. The members of this brood were considerably younger than those forming brood *A*. There were eight embryos in brood *C*, nine in brood *D*, eight in brood *E* and at least six in brood *F*. It is possible that

a still younger brood was present. The largest unfertilized cells were about .7 mm. in diameter and it is evident that fertilization would occur when the cells had reached this size.

The only peculiarity in the history of the ova up to the time of fertilization lies in the fact that they are very much smaller at fertilization than the ova of the other species described. This fact would indicate that the larger quantity of yolk elaborated in the ova of the others is not required in *Heterandria formosa*. If the larger amount of yolk is not formed and yet the embryos go on to the more advanced stage while being retained in the ovary, it must be assumed that the follicle cells surrounding the developing embryos are responsible for furnishing the food materials. The expanded yolk sac, containing little yolk, is in a position to absorb the food materials, for it completely surrounds the embryos in the earlier stages, becomes very vascular, and is in contact with the walls of the follicle almost up to the time of birth.

Both viviparity and superfetation in this species have been carried to an extreme degree and it is quite likely that there will be found in some small pœciliid fish a stage intermediate between that of *Heterandria formosa* and that represented by *Pœciliopsis infans* and *Pœcilistes pleurospilus*.

It is reported by Stoye (1935) that the mode of reproduction in *Priapella bonita* and in *Phalloptychus januarius* is similar to that in *Heterandria formosa*, one or two young being born at a time at intervals of a few days.

It is also reported by Stoye that in *Micropœcilia branneri* the young are born one or two at a time every few days. This observation would tentatively class this species with *Heterandria formosa* in its mode of reproduction. A most interesting situation is presented in this case since it has been stated by Stoye and verified by the writer that two other species in this genus (*M. parœ* and *M. picta*) produce broods of thirty to forty embryos at intervals essentially like those of the *Lebistes* type. Unfortunately no breeding specimens of *Micropœcilia branneri* have been available and gravid ovaries have not been examined.

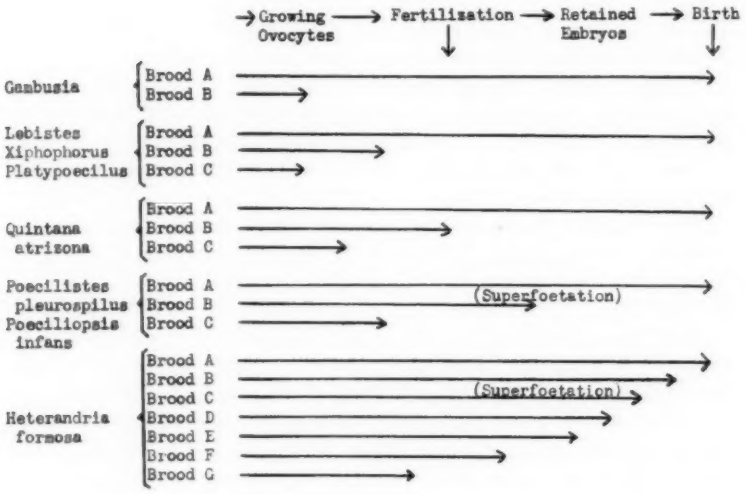
Summary

A graphic summary of the relations of growing ovocytes, mature ova, the fertilization process, the broods of embryos retained in the ovary and the time of birth is offered in Table IV. Each group represents the situation within a single ovary at the height of the reproductive season and at the moment in which a brood is born. The term brood is used to indicate a group of growing and differentiating ovocytes of approximately equal development up to the time of fertilization and also the

embryos produced by the fertilization of these ova up to the time for birth. No quantitative relations are implied concerning comparative size of ovocytes or embryos in different species nor of comparative lengths of time that embryos are retained in the ovary.

A study of Table IV leaves one with the impression that the extreme type of superfetation in *Heterandria formosa* might have arisen from the simpler type as illustrated in *Pœcilistes pleurospilus*. The argument also appears to be defensible that the simpler type of superfetation found in *Pœcilistes* might have arisen from a condition like that illustrated by *Quintana atrizona* in which a new group of ova are ready for

TABLE IV



fertilization when a brood is born but in which fertilization is delayed for some hours. All of this implies that the extreme cases of superfetation should be considered as the specialized and derived types while those without superfetation are the simpler. Since the Pœciliïdæ undoubtedly arose from an oviparous cyprinodont such an assumption does not seem to be overdrawn. That group of the Pœciliïdæ (*Gambusia* and *Mollienisia*) with the ovarian cell cycle which most resembles the oviparous cycle may by the same sign be considered the most unspecialized.

The evidence which now exists concerning the evolutionary relations of the different genera of the Pœciliïdæ gives no support to the thought

that those genera in which superfetation occurs are more closely related to each other than to any other genera. Rather, it appears that superfetation has arisen more than once and that the present cases represent convergent evolution. The occurrence of superfetation therefore has no taxonomic significance.

The Control of Reproductive Cycles

The factors which control the rhythms of reproduction in this group of fishes are presumed to be the same as for other fishes and are probably also the same group of factors which control the cyclical production of ova in higher vertebrates. These factors would be in part environmental, in part the driving influence of the gonad-stimulating hormones of the pituitary and, finally, an hereditary factor which would place a limit within which the others might act.

Annual Rhythm

In most of the laboratory-reared pœciliids in which an annual record has been kept, it has been noted that the number of embryos in a brood and also the number of broods produced increases in early spring, reaches a climax in late spring and early summer and declines in late summer. Production of young falls to a low level or ceases altogether during the winter. Studies on *Gambusia affinis* by Barney and Anson (1921*a*), Hildebrand (1917) and Kuntz (1913) show that the same type of rhythm is present in specimens studied in the field under natural conditions. In the laboratory specimens reared by the writer, food and temperature have been maintained at a constant level. In the natural habitat, both temperature and food supply have some effect on the rhythm. The laboratory-reared specimens have been subjected to one variable factor, namely light. No attempt has been made to regulate the length of time each day during which specimens were exposed to light and consequently they have been exposed to the usual seasonal fluctuation. The highest point in the reproductive cycle coincides with the longest daily exposure to light and the lowest point in the reproductive rhythm to the shortest daily exposure. This coincidence would seem to be significant in view of the fact that seasonal variation in light has accompanied seasonal variation in the reproductive cycle in both wild and laboratory specimens. It might be tentatively postulated that light acts directly to stimulate the reproductive organs differentially but more likely through the gonadotropic hormones of the anterior pituitary.

Brood Production

Apparently the factors which control the size of broods and the rate of brood production are in part the same as those concerned with the high and low phases of the annual cycle. The contrast between the reproductive activity exhibited by an ovary of *Heterandria formosa* at the height of the reproductive season and one at a low ebb is striking and demonstrates clearly one of the factors responsible for brood production. In the ovary at the height of the breeding season there are five or six broods of several individuals each at different levels of development. At a still lower level are one or two groups of cells growing to the size necessary for fertilization. Every few days a brood is born and all levels of embryos and younger fertilized cells move up to a higher level. The entire picture is one of the crowding up of the maximal number of cells from the lower levels with a stimulating factor driving them, with the result that larger broods are produced and the intervals between them decreased. The reverse of this picture is seen in the ovary of specimens of this same species during the winter. Small numbers of ovocytes are developing and if broods are present in the ovary there are rarely more than two and they consist of one or two embryos each with a wide gap between them in level of development. The impression is created that the cause for the infrequency of broods, the small numbers of embryos in broods, and the paucity of developing ovocytes, lies in the lack of a stimulating agent. As suggested before this agent is probably a follicle-stimulating hormone from the anterior pituitary.

An influence retarding the development of the younger ovocytes is also present. This is best seen in the ovarian activity in species without superfetation. In *Gambusia,* for example, the younger ovocytes will develop to a diameter of only .3 mm. until the brood of embryos held in the ovary is released. Then within a few days they more than triple their diameter. In *Lebistes* and *Xiphophorus* the retarding effect is not so marked and ovocytes become much larger (0.8 mm. to 1.0 mm.) before a brood is discharged. In *Quintana atrizona* retardation by the growth of embryos is still less and the ova reach the maximal size and are ready for fertilization before the previous brood is evacuated. However, the retention of a brood of embryos seems to act as a block upon fertilization; otherwise some cases might be found in which the fully matured ova had been fertilized before the discharge of the older brood of embryos. Although the block upon fertilization of a new group of ova exercised by the presence of a brood of embryos seems to be removed in *Pœcilistes pleurospilus,* it is apparently partly operative

for not more than two levels of broods are found in the ovary at one time.

The presence of older ova also serves to retard the development of younger cells. In an old virgin female a group of ova will have reached the maximal size and younger groups of cells approximately equal in numbers will be found at lower levels. If fertilization is prevented, the younger groups of cells will not develop and the condition may prevail for months. When the older group of ova is fertilized and the embryos begin to develop, the block is released and when the embryos are discharged the younger waves of cells will advance.

From the above observations the controlling mechanism might be postulated as follows: (1) a hormone stimulates the ovary to produce waves of developing ovocytes. The production of the hormone or its effectiveness is acted upon by light and possibly other factors in the environment. (2) A retarding influence upon the growth of younger cells is exercised by the presence of older cells and by broods of retained embryos. (3) A balance has been struck between the two influences and is fairly constant within a species so that the rate of brood production at any season is practically constant. Hereditary factors control this balance. (4) The balance between the stimulating and retarding influences varies with the season, the stimulating agent being most effective in spring and early summer and least operative during the fall and winter months.

Relation of Broods to Annual Cycles in Other Fresh Water Fishes

Some of the fresh water fishes of the temperate zones have annual life cycles, maturing their gametes within the year and dying before another season of reproduction. This type of life cycle, described by Hubbs (1921) in *Labidesthes sicculus,* is not common. Other fishes bring their ova to maturity at the end of their second or third year or later and a new generation of ova to maturity each year thereafter as long as they live. In some fishes the ova have been two years in coming from ovogonia to the full size and complete differentiation necessary for fertilization. *Cottus bairdii,* described by Hann (1927), represents this type. It has been observed by the writer that *Perca flavescens* in Lake Michigan follows the same course in developing its ova although there is evidence that *Perca* requires sometimes three years before the first batch of ova is matured. In these fishes of the temperate zone a marked retarding influence is exerted by the cold weather of late fall and winter. Under more favorable food and temperature conditions such as can be

maintained in the laboratory, an earlier maturing of gametes can be secured indicating that the reproductive rhythm occurring in nature is not the maximal one. These same fishes under tropical conditions would presumably produce more young in a shorter space of time although inherent factors would limit the rapidity of the occurrence of cycles.

As already indicated, the Pœciliïdæ arose in a tropical climate (Eigenmann, 1906) and some genera have migrated into the north and south temperate zones. Their pattern in reproductive cycles has apparently been fixed in the tropics under most favorable conditions for the production of the maximal number of cycles in the shortest space of time. Apparently other inherent factors associated with viviparity have had some influence in shortening reproductive cycles as oviparous fishes like the cichlids, which have been associated for a long time with the Pœciliïdæ in geographic distribution and climatic conditions, do not have such short cycles.

The production of successive waves of ovocytes in the Pœciliïdæ is interpreted as parallel to the seasonal waves produced in such forms as *Cottus* and *Perca*. It takes two years to produce a batch of mature ovocytes in *Perca* and *Cottus* and only a few months in the pœciliïds. If any pœciliïd should produce a single batch of ova, bring them to maturity and fertilization and void the embryos without bringing any more ovocytes to maturity, we should have a case parallel to that of *Labidesthes*, but apparently none exists. The interpretation of each pœciliïd short cycle as equivalent to the long cycle of *Cottus* and *Perca* instead of a long-drawn-out annual cycle, as suggested by Barney and Anson and Kuntz, rests upon the question of whether there is synapsis in residual gonial cells on a large scale to produce the entire season's potential ova or synapsis of only enough residual gonia to form the ova for a single brood. If synapsis occurred on the necessary large scale and only once a year, followed by the maturing of small groups of ovocytes, an annual cycle such as occurs in *Cottus* and *Perca* might be postulated. However, during the height of the breeding season small waves of synapsis of gonia occur at regular intervals and new ovocytes are being added at the lower levels as the ones at the upper levels reach maturity and are fertilized.

Summary

1. All species of the Pœciliïdæ are ovo-viviparous.

2. At the height of the breeding season broods are produced at regular intervals varying from about forty-five days in some species to five or six days in others.

3. In laboratory-reared specimens and in specimens breeding in temperate zones the shortest interval between broods occurs in spring and early summer and the longest interval during fall and winter.

4. In the reproductive cycle of *Mollienisia* and some other species, the oldest group of ovocytes remaining in the ovary just after a brood has been extruded is very small. Growth is rapid and the ova are ready for fertilization about eight days after the voiding of the last brood of embryos.

5. In *Lebistes* and other species with similar reproductive cycles, the oldest group of ovocytes remaining in the ovary just after a brood has been voided are much larger than in *Gambusia* and fertilization occurs a few days earlier than in *Gambusia.*

6. In *Quintana atrizona* the oldest group of ova remaining in the ovary are fully developed and ready for fertilization at the time of birth of the previous brood of embryos.

7. Superfetation occurs in *Pœcilistes pleurospilus* and several other species. Fertilization of the oldest group of ova takes place before the voiding of the brood already in the ovary and two broods at different levels of development are found in the ovary.

8. In *Heterandria formosa* and possibly other species there is an extreme development of superfetation with six or more small broods at different levels of development occurring in the ovary at one time at the height of the breeding season.

9. Older groups of ova and embryos retained in the ovary retard the development of younger groups of cells and a balance is maintained between this retarding influence and the agent, assumed to be a follicle-stimulating hormone from the pituitary, which forces the cells to grow from the lower to the upper levels of development.

10. The short reproductive cycles of the Pœciliïdæ are the equivalent of the longer annual or biennial reproductive cycles of the fishes of the temperate zones.

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THE EXPERIMENTAL DECOMPOSITION AND REGENERATION OF NITROGENOUS ORGANIC MATTER IN SEA WATER[1]

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Historical

Although the formation of nitrate from composted nitrogenous organic materials has been long known and practically applied, the current conception that specific biological agencies bring about these natural processes in soils, fresh waters, and the sea was developed only in the last quarter of the nineteenth century. In his studies on acetic acid fermentation Pasteur (1862) anticipated the rôle of bacteria in the ultimate oxidation of ammonia to nitrate, but Schloesing and Müntz (1877 et seq.) first demonstrated the biological nature of the intermediaries. The demonstration was performed in soils; Müntz, shortly afterward (1890), showed that ammonia was produced in soils by microbial breakdown of proteinaceous organic matter. That the oxidation of ammonia to nitrate proceeded through nitrite was distinguished by Munro (1886), but not until Winogradsky (1891, 1892) performed his classic researches were the two oxidations recognized as separately determined by specific ammonia-oxidizing and nitrite-oxidizing bacteria.

Following these researches Adeney (1895) demonstrated the complete sequence of nitrate regeneration in natural and sewage polluted water, establishing the steps by clear-cut quantitative methods. His procedures determined the basis for standard sewage analysis.

It has been held by Brandt (1899, 1902) and many others that the cycle of nitrogen in the sea follows essentially the same phases, and, using this interpretation, a number of researches into the occurrence and distribution of specific marine organisms have been made. These investigations have been reviewed and extended by Waksman, Hotchkiss and Carey (1933). There is considerable conflict in the literature on biological nitrification in the sea, but the conventional picture derived from studies of soils and fresh waters is nevertheless applied to oceanographic chemical data.

[1] Contribution No. 129 of the Woods Hole Oceanographic Institution.

Experimental

Because of the growing interest in the regeneration of nitrate in the sea it seemed highly desirable to make a quantitative investigation of the course of organic decomposition and of the simultaneous appearance of nitrogenous products in the water. To this end, a number of experiments were carried out in which a natural source of organic matter—plankton material from customary net-hauls—was allowed to rot and

Table I

Series I. Source of organic matter: mixed plankton tow.
Micrograms of nitrogen per liter.

Date	Plankton	In water					Total in system ‡	Dissolved organic N	Bacteria in thousands per ml.
		Ammonia	Nitrite	Nitrate + nitrite	Nitrate	Total in water			
6–27	188	0	0	5	5	5	193	150	100
29	150								160
30		80	0	5	5	85			204
7– 2	131	107	0.1	5	5	112	243		180
5	110	160	3.8	6	2	166	276		123
10	82						277		
11		185		10		195			22
15			2.4						
17	77						308		40
18		225	2.7	6	3	231			
24	40		16				272		
25		200		32		232			20
27			55	70	15				
28		185	80	90	10	275			
30		125	110	145	35	270			36
8– 1		60	180	215	35	275			
5		11	150	275	125	286			
7	47	0	110	300	190	300	347		
10		8	2.2	300	298	308			
8–11*		Portion inoculated with diatoms							
19	285	0	0	20	20	20	305		
22	300	0		13	13	13	313		
8–11*		Control portion, not inoculated							
12			0.5	360	360				
14				330					
17			0.6	330	330				
19	37	0		360	360	360	397	200	
21			0.8	330	330				
22		0							

* One portion inoculated with diatoms and placed in the light.
‡ Does not include dissolved organic nitrogen.

decompose in sea water. Observations were made to follow the disappearance of nitrogen in the decomposing material and its appearance in the water in the form of ammonia, nitrite and nitrate, in the effort to reproduce artificially the cycle of organic decomposition and eventual regeneration. If, by means of the soluble compounds resulting from the decomposition, the water could be rendered fertile and capable of

TABLE II

Series II. Source of organic matter: mixed plankton tow.
Micrograms of nitrogen per liter.

Date	Plankton	In water					Total in system ‡	Dissolved organic N	Bacteria in thousands per ml.
		Ammonia	Nitrite	Nitrate + nitrite	Nitrate	Total in water			
7–12	400	18	0.2	8	8	26	426	420	82
14	345								176
15		>120	0.5	8	8				265
17	275	>120	0.2	6	6				270
20	202	550	2.3	8	6	558	760		80
23		580							
24			16						43
25		600		27		627			
27			60	80	20				
28		650	110	150	40	800			25
29	90								
30		550	240	300	60	850	940		
8– 1		340	550	590	40	930			16
5		12	800	820					
7	78	4	850		60	914	992		
8				910					
10		8	550	1000	450	1000			20
12			190	1100	910	1100			
14		35	0.1	1050	1050	1100			25
17			0.1	1100	1100				
18	133						(1250)		
8–21*		Portion inoculated with diatoms							
27	305								
28			1.0						
31	840		7.7	320	312	(330)	(1170)		
9– 2	1010		0	25	25	(35)	(1045)		
3								470	
8–21*		Control portion, not inoculated							
22		7	0.5						
28			0.3	1050	1050				
29				1000					
31			0.5						

* One portion inoculated with diatoms and placed in the light.
‡ Does not include dissolved organic nitrogen.

supporting a new organic growth, comparable to that previously decomposed, the complete cycle would have been carried out. It was also hoped that periodic chemical analysis, by methods now available, might throw light on the sequence of the various steps in the cycle.

The raw material was mixed plankton, collected in the usual way in a No. 20 net, quickly washed, and kept on ice for the few hours before it could be returned to the laboratory. This material was then suspended in 10–15 liters of fresh, filtered sea water and stored in the dark at a temperature of 20–25° C.

Several different analyses were carried out on this material at the start and at intervals thereafter, the various fractions being determined as follows:

1. Total nitrogen contained in the suspended or particulate matter. This is called "plankton nitrogen" in the following descriptions, but also includes bacterial nitrogen as well as that in any other form of suspended matter. This determination follows the procedure described by von Brand (1935) and consists essentially of precipitating the

TABLE III

Series IV. Source of organic matter: mixed plankton tow, strained through No. 8 bolting silk. Micrograms of nitrogen per liter.

Date	Plankton	In water					Total in system *	Bacteria in thousands per ml.
		Ammonia	Nitrite	Nitrate + nitrite	Nitrate	Total in water *		
7–28	115	43				55	170	152
29			0.6	12	12			200
30	83	110						305
8– 1	63	150	1.0	15	14	165	228	190
5	38	205	<1	10	10	215	253	35
7		190	0.6	10	10	200		16
10	40	200		15	15	215	255	
14		240	0.5	20	20			25
17	41			17	17			
20			13					
21				35				20
22		210	21			(250)		18
27	58	175	120	140	20	315	373	
29			190					
31		29	220	240	20	269		
9– 2		25	225	225	(0)	250		
5	59							
8				260				
14		14	310	330	20	344	(400)	
27			130	290	160			
10–4			1					

* Does not include dissolved organic nitrogen.

suspended matter in a sample of the water by the addition of alkali. The precipitate drags down all suspended and colloidal matter, and after settling and centrifugation is separated and resuspended. The resulting small volume of suspension is then used for a determination of total nitrogen by the method of Krogh and Keys (1934): fusion with KOH in a stream of hydrogen, followed by recovery of the ammonia.

2. Ammonia in the water, by a slight modification of the method of Krogh (1934), in which the original design of the still was changed somewhat, to make it more compact and to permit heating electrically.

3. Nitrite in the water by the well-known Griess-Ilosvay method.

4. Nitrate (including nitrite) in the water, by Harvey's reduced

TABLE IV

Series VI. Source of organic matter: washed, persisting culture of *Nitzschia*. Micrograms of nitrogen per liter.

Date	Plankton	In water					Total in system *
		Ammonia	Nitrite	Nitrate + nitrite	Nitrate	Total in water *	
8–14			0.1				
15	633	2		17	17	19	652
18	514	120	0.1	15	15	165	679
21	430	230	0.1	16	16	246	676
27	298	310	0.4	24	24	334	632
9– 2	201	410	1.3	20	19	430	631
8	226			25	25		
14		490	0.1	17	17	507	(700)
27			0.2	20	20		

* Does not include dissolved organic nitrogen.

strychnine method. Nitrate then calculated by subtracting the value of nitrite as obtained under 3.

5. Bacterial counts.

The data from the chemical analyses are collected in Tables I to IV and some of them are also presented graphically in Figs. 1 and 2.

Several different "series" were carried out: I, II and IV, in which the organic material was a mixed tow of both phyto- and zoöplankton, of varying amount in each case; and VI, in which a persisting washed culture of diatoms was used as the source of organic matter. In Series II the sea water in which the plankton was suspended came from the surface of Woods Hole Harbor, which probably accounts for its higher content of dissolved organic nitrogen than that of the water in

the other series, which was obtained from the surface a few miles to the south of Marthas Vineyard.

Chemical

The rate of decomposition of the plankton varied in the different series, but after an interval of from 8 to 20 days decomposition stopped. At this time there was still a constant and not inconsiderable amount of nitrogen still remaining, either in non-decomposable plankton residues, bacterial cells, or in other forms. The amount of this residual nitrogen was from 20 to 35 per cent of that originally determined in the plankton, or 7 to 10 per cent of that calculated by addition of the different soluble and insoluble nitrogen fractions.

The rate of decomposition is greatest in the first few days. In Series II, IV and VI it dropped rather suddenly, but in Series I it fell gradually over a longer period of time.

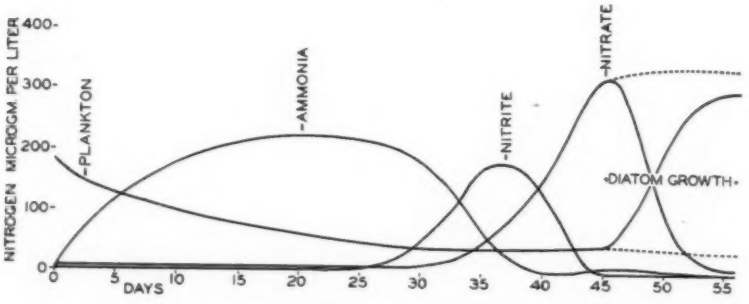

Fig. 1. Series I. The decomposition of nitrogenous organic matter in mixed plankton, showing the appearance of soluble nitrogen compounds in the water in which it is suspended. One portion inoculated with diatoms on the forty-fifth day.

Ammonia appeared in the water rapidly and immediately at the beginning of the decomposition. Evidently the liberation of ammonia is involved in the very first steps in the degeneration of organic matter. It appears more slowly as time goes on, and reaches a maximum when the plankton decomposition ends. During this time there is no rise in the nitrite or nitrate in the water.

The disappearance of ammonia is accompanied by the appearance of nitrite in the water, and this in turn is eventually oxidized to nitrate. The phase during which nitrite was present was 15–20 days in Series I and II, but extended to 40 days in Series IV. In any event, it is not until nitrite reaches its maximum and starts to disappear that nitrate increases.

Eventually, however (after 45 days in Series I, 40 days in II, and 65 days in IV), all the available nitrogen was oxidized to nitrate. At this point, in both Series I and II, a portion of the water was placed in the light and inoculated with fresh diatoms, which grew rapidly, raising the " plankton nitrogen " again and lowering the nitrate to its original minimal value. In this way the complete cycle was carried out.

The question of the existence of soluble nitrogen compounds intermediate between plankton material and ammonia could not be definitely answered, but there was no evidence of any such. Anything in the nature of colloidal cell-material, partially decomposed, must have been carried down in the precipitation and determined with the rest of the plankton nitrogen.

In the transformation of ammonia to nitrite and nitrate it seems that we are dealing not with an equilibrium of simultaneous processes but with distinct, consecutive steps, each doubtless determined by its own set of conditions.

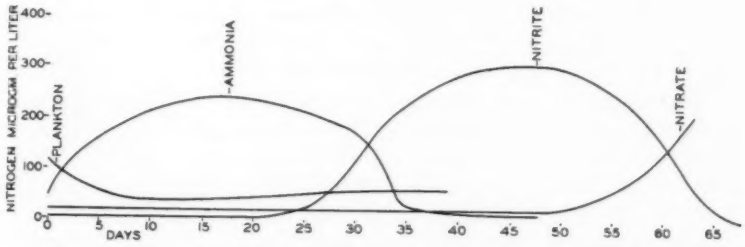

FIG. 2. Series IV. The decomposition of nitrogenous organic matter in mixed plankton, showing the appearance of soluble nitrogen compounds in the water in which it is suspended. Plankton previously filtered through No. 8 bolting silk.

A peculiar situation is revealed by the data from Series I, II and IV. The analyses indicate a larger amount of nitrogen regenerated in soluble forms in the water than that lost by the plankton during decomposition. In the tables the " total nitrogen in the system " has been calculated by addition of all the separate forms determined. This total shows in general a continuous increase throughout the decomposition, especially in Series II. That it is not observed in Series VI may be in some way connected with the fact that the organic material in this case consisted of diatoms.

There are three possible explanations for this nitrogen discrepancy:

1. Participation of other forms of nitrogen in the cycle—most plausibly the dissolved organic nitrogen in the water.

2. Systematic errors in either the sampling procedure or the analytical method, so that the values for plankton nitrogen are regularly too low.

3. Nitrogen fixation.

Each of these possibilities has been critically examined and at the present time none of them seems an altogether acceptable explanation. Determinations of the dissolved organic nitrogen in the water, by the method of Krogh and Keys, did not show any significant change during the experiments. Filtering out the larger plankton organisms in Series IV, to lessen the chance of irregular sampling, had no effect. On the other hand, it is true that the decomposition of very much more finely divided organic matter (diatoms) in Series VI yielded only an equivalent amount of ammonia, but this experiment had to be stopped before nitrification began, which is usually the time when the most pronounced difference between added and recovered nitrogen appears.

According to currently accepted views of biological fixation of nitrogen the third possibility seems doubtful.

A further study of this nitrogen discrepancy is now under way.

Bacteriological

The regeneration of inorganic nitrogen from its various organic forms in plankton proceeds through four recognizable stages, each of which is incident to the development and activity of a special bacterial flora. Following the death of plankton organisms, brought about in these experiments by handling, unfavorable temperatures, lack of illumination, crowding, and inadequate nutrient supply, the more labile components of the dead cells undergo hydrolytic cleavage, simultaneously liberating a fraction of the amino nitrogen as ammonia. This breakdown, it would appear from the rate at which ammonia appears and plankton nitrogen diminishes, is not analogous to the digestion of protein by higher animals where individual protein complexes are first split into smaller molecules bearing amino nitrogen.

A part of the decomposition may be autolytic, but it is evident from the tables, which show an attending rapid development of various species of bacteria, that these organisms must play a large part. The absolute numbers are never very high—in fact, not greater than would be expected in freshly taken, unfiltered sea water stored for such intervals of time (Waksman and Renn, 1936; ZoBell and Anderson, 1936). The decline in plankton nitrogen is not so precipitous as the decrease in bacterial numbers. This may mean that the various fractions of plankton nitrogen are not equally susceptible to bacterial attack.

Liberation of ammonia from plankton and other nitrogenous organic matter is, bacteriologically speaking, unspecialized, but is dependent upon the relative proportions of available nitrogen and carbon in the decomposing materials—the materials used in these experiments are, as Redfield (1934) has shown, characteristically nitrogen-rich.

Following the first burst of bacterial activity the ammonia concentration reaches a high level capable of supporting an active nitrite-forming population. Unsuccessful attempts were made to follow the development of the specific bacterial flora responsible for the oxidation of ammonia to nitrite. It appears from the many investigations upon nitrite-forming bacteria in the sea, that the process is due to specific organisms of the *Nitrosomonas* group analogous to the specialized nitrite-forming flora of soils (Waksman, Hotchkiss and Carey, 1933; Carey and Waksman, 1934). The rate at which nitrites are developed here is roughly the same as that observed in cultural studies of the process. Crude cultures prepared by inoculating sea water containing ammonium salts with mud or sea water usually give positive tests for nitrite within ten days or two weeks (depending upon the quantity of inoculum), after which the complete oxidation of ammonia to nitrite proceeds rapidly.

The final stage in nitrogen regeneration progresses more slowly, as is evident from Figs. 1 and 2. Nitrate formation is also effected by a specific bacterial flora, in this case, *Nitrobacter,* an efficient population of which seems to develop at a lower rate than either the non-specific ammonifying or specialized nitrite-forming bacteria. In culture experiments where crude inocula of nitrate-forming bacteria are added to sea water containing nitrite as the only sources of nitrogen, the period of incubation necessary to demonstrate measurable quantities of nitrate ranges from 30 to 90 days. Richer inocula bring about more rapid nitrate formation (Waksman, Hotchkiss and Carey, 1933).

It has been assumed that the relatively late appearance of nitrate in cultures made up of raw sea water to which organic nitrogen in some form has been added is due to the toxic effect of ammonia on nitrate-forming bacteria—a condition that has been clearly demonstrated in experiments on soils. But this relation, if it exists in these experiments, is masked by the slow growth of nitrate-forming bacteria even under favorable conditions. Thus in Series I and II the phase of nitrate formation began promptly after the disappearance of ammonia; in Series IV, on the other hand, it did not begin until 15 days after the ammonia had disappeared.

The regeneration experiments described here certainly are not identical with natural conditions and may not be expected to parallel exactly the courses of the individual phases of nitrate formation in the sea. On

the other hand, they offer a clear picture of their relations. Further, they validate the conventional conception of the nitrogen cycle as derived from the various studies of the individual processes, by bringing them together in a single summary experiment.

Summary

In conclusion, this study has shown:

1. That it is possible to reproduce the complete cycle of nitrogen regeneration.

2. That the transformation of decomposing plankton (especially diatoms) into ammonia is very rapid, beginning as soon as the initial substance disappears from the body. The amounts of soluble nitrogenous substances of higher molecular weight in these experiments can have been only very small.

3. The main stages in the decomposition are: dead body—ammonia —nitrite—nitrate.

4. Under these conditions, at least, no toxic substances are formed which inhibit the flowering of diatoms. The rapid and abundant development of the latter showed the regeneration of the water to be complete.

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STUDIES IN THE PIGMENTARY SYSTEM OF CRUSTACEA

II. Diurnal Movements of the Retinal Pigments of Bermudan Decapods

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Diurnal rhythms in behavior and physiological rhythms in certain organ systems of animals have long intrigued biologists and led to much interesting speculation as to the nature of the mechanisms involved in such activities. That the phenomenon is a general one is seen from the cases cited by Welsh (1930*a*). Such periodicity is most strikingly exhibited in the pigmentary changes of various animals, being reported in shrimps by Gamble and Keeble (1900), in brachyurans by Megušar (1912), in isopods by Menke (1911), in amphibians by Slome and Hogben (1929), in reptiles by Redfield (1918), and in cyclostomes by Young (1935). It is reasonable to expect that further study will reveal the existence of such rhythms in additional forms. Welsh (1930*a*, 1935, 1936) has shown that diurnal changes in the migration of the retinal pigments occur in several crustaceans even though the animals are maintained under constant conditions of illumination or of darkness.

In all the cases mentioned hormonal factors are involved to a greater or lesser degree in controlling pigmentary activity. Since the present studies (Kleinholz, 1937) are concerned with chromatic responses in crustaceans, only this branch of the general subject of color changes will be discussed here. The control of the bodily changes in color in crustaceans has been shown by Perkins (1928) and by Koller (1928) to be maintained by an endocrine which is liberated into the circulatory system from within the eye-stalks. Hanström (1935) and his associates (Sjögren, 1934; Carlson, 1936) have made histological studies of the eyes of many crustaceans, and have correlated the activity of the chromatophorotropic principle in eye-stalk extracts with the presence within the eye-stalk of a secretory structure which they call the *blood gland*. The mechanism that is involved in the control of retinal pig-

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ment migration in crustaceans is not yet completely known. Recent studies on *Palæmonetes vulgaris* (Kleinholz, 1936) present evidence for a humoral control of the distal and the reflecting pigments in the retina, but the proximal pigment appears not to be affected by the same eyestalk extracts which activate the first two sets of pigments. It is possible that one set of pigment cells is under nervous and the others under hormonal control.

Before much progress can be made in analyzing the mechanisms by which diurnal rhythms are maintained under constant external conditions, it is advisable to study the phenomenon in a number of different species to determine which combinations of retinal pigments may be involved in such activities. This report on diurnal changes in the retinal pigments of several Bermudan crustaceans is offered to this end.

Materials and Methods

The various crustaceans used in these experiments were obtained by dredging at several stations in the vicinity of the Biological Station. After a sufficient number of animals of the same species had been brought into the laboratory, the specimens were divided into two groups, one of which was placed in a white porcelain bowl illuminated by a 40-watt electric lamp at a distance of 18 inches, while the second group was placed in a container in the dark-room. At least 12 hours were allowed for individuals to become adapted to light and to darkness.

After a period appropriate for adaptation, specimens were removed for fixation of the retinal pigments. For purposes of description the term " day-light " eye is used to indicate the retina of a specimen that was kept constantly illuminated and which was fixed or examined in the daytime, while " night-light " eye represents the condition of the pigments in an illuminated retina that was fixed at night; conversely, " day-dark " and " night-dark " are used to designate those specimens maintained in constant darkness whose retinal pigments were fixed during the day and at night, respectively. When a specimen was taken from the light-adapted group, a similar specimen was removed from the container in the dark-room for fixation at the same time.

Two methods of fixation for histological study were used. In one method, the animals were dropped into hot water (80° C.) for 10–20 seconds to fix the positions of the retinal pigments, and were then tranferred either to 5 per cent formalin or to a modified Bouin's solution containing 7 per cent acetic acid. In the second method the entire specimen, after the usual period of adaptation to darkness or to light, was dropped into a vial of Bouin's solution. The exoskeleton of most of the crustaceans contained large amounts of calcareous salts, but these

troublesome deposits were dissolved by the acetic acid in the fixative, so that sectioning the eyes was greatly facilitated.

When the exoskeleton had been sufficiently softened by the fixative, the eye-stalks were excised and embedded in paraffin by a rapid dioxane treatment. After a preliminary rinsing of the excised eyes in water, the stalks were rolled over filter paper to remove any excess moisture

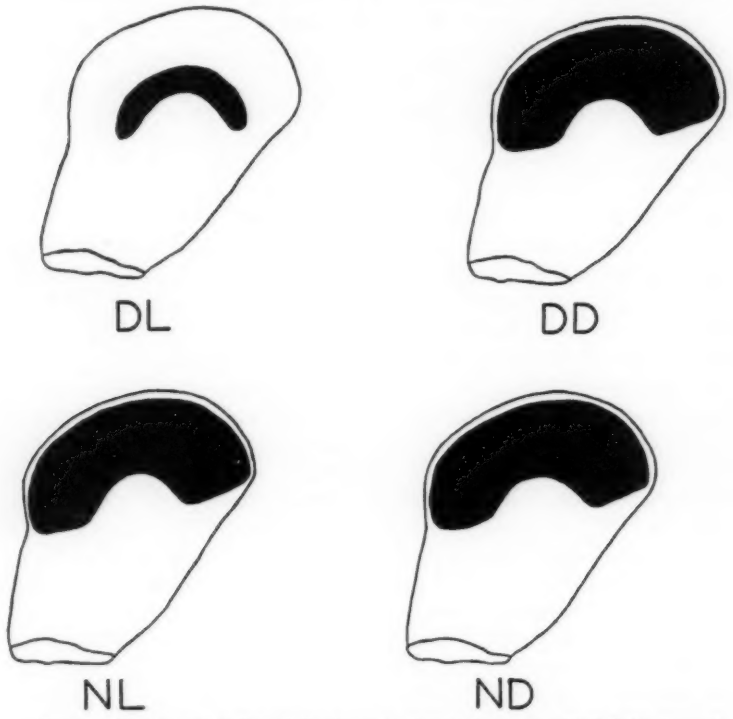

FIG. 1. Entire eye-stalks of *Eusicyonia*, viewed by transmitted light through the low powers of the microscope and showing the positions of the distal retinal pigment. *DL*, from a day-light animal; *DD*, from a day-dark specimen; *NL*, from a night-light individual; *ND*, from a night-dark shrimp.

and were then placed in full-strength dioxane over anhydrous calcium chloride. They were left in this fluid usually overnight (8–10 hours) and the next morning were placed directly into soft paraffin. The tissues were allowed to become infiltrated with the wax for about three hours, using two changes of paraffin, and were then embedded. No difficulty was encountered in cutting serial sections at 10 micra. Some

sections were subsequently stained with Delafield's hematoxylin and eosin, and others were mounted unstained.

I am indebted to Mr. M. D. Burkenroad of the Bingham Oceanographic Foundation at Yale University, and to Dr. Fenner Chace, Jr., of the Museum of Comparative Zoölogy at Harvard University for identifications of the collected crustaceans. The two macrurans studied were *Eusicyonia n. sp.* (to be described by Mr. Burkenroad) and *Trachypeneopsis mobilispinis,* while the brachyurans were *Portunus anceps, Portunus depressifrons, Parthenope serrata,* and *Calappa flammea.*

Eusicyonia n. sp.

This new species of shrimp was taken from dredgings at 10–15 fathoms off Murray's Anchorage. Of the 12 specimens obtained for experimental study, 2 were males and the rest females.

While preliminary observations of the eyes of specimens kept under constant illumination showed that there was a change in position of the distal retinal pigment at night (Fig. 1), sectioning the eye was necessary to disclose the photomechanical changes of the remaining pigments. The eye of *Eusicyonia* is similar in structure to that of *Palæmonetes* as described by Parker (1897) and by Welsh (1930*b*). The positions of the three types of pigment cells are shown in Figs. 2–5.

An unusual situation was discovered in studying a number of sectioned eye-stalks of this species. Of the 12 specimens at hand, the retinas of 4 showed complete absence of reflecting pigment. The possibility that this might be a secondary sexual difference is eliminated by the fact that 2 of these shrimps were males and 2 females. The possibility that the difference is a fixation artifact can be ruled out by comparing eyes of specimens fixed by the two different methods. The reflecting pigment in the integument of many of the lower vertebrates is guanin, and is commonly found there in the crystalline condition (Ewald and Krukenberg, 1882). So far as I know, however, no studies have been made on the chemical nature of the pigments in the crustacean retina. If the retinal reflecting pigment is guanin, it might be slightly soluble in the acid components of the Bouin's solution. But the pigment was lacking only from those eyes which had been fixed with hot water and then preserved in formalin. This treatment does not seem to be sufficiently harsh to effect such changes. Moreover, of 4 specimens of *Trachypeneopsis* which had been fixed by the same method, the eyes of 1 lacked this reflecting pigment, while those of a second specimen were slightly deficient (Fig. 15); the retinas of the other 2 animals showed what may be called "typical" amounts. It seems more likely that the differences in amount of reflecting pigment are due to

individual variations, possibly in the nucleoprotein metabolism of the animals, than to any fixation artifact.

On studying sections of the eyes of *Eusicyonia* fixed under the four experimental conditions (Figs. 2–5, 10–13) the following situation is found in the retinal pigments. The granules of reflecting pigment undergo no apparent changes in position in response to changes in light intensity. The main mass of the reflecting pigment is located on the distal face of the basement membrane; smaller amounts are found below the basement membrane and capping the distal ends of the distal retinal cells. The distal and the proximal pigments show readily discernible photomechanical changes:

Day-light eye

A. The distal pigment cells are to be found in the typical light-adapted state, the cells having moved proximally and come to rest against the proximal retinular cells.

B. The proximal pigment is also in the position typical for the light-adapted retina, much of it having migrated above the basement membrane to surround the rhabdome.

EXPLANATION FOR PLATE I

All figures are camera-lucida outlines to insure correct proportions, but the details have been drawn diagrammatically. *BM*, basement membrane; *DD*, day-dark retina; *DL*, day-light retina; *DP*, distal retinal pigment; *ND*, night-dark eye; *NL*, night-light retina; *PP*, proximal retinal pigment; *RP*, reflecting retinal pigment; *R*, rhabdome.

FIG. 2. Ommatidium from a day-light eye of *Eusicyonia*. The distal pigment lies against the proximal retinular cells, while much of the proximal pigment has migrated above the basement membrane to surround the rhabdome.

FIG. 3. Ommatidium from a day-dark retina of *Eusicyonia*. The pigments are in the typical dark-adapted position, the distal pigment having migrated distally towards the cornea, and the proximal pigment having moved completely below the basement membrane.

FIG. 4. Ommatidium from a night-light eye of *Eusicyonia*. The distal pigment is in the position characteristic for the dark-adapted retina while the proximal pigment is found above the basement membrane.

FIG. 5. Ommatidium from a night-dark eye. Both pigments are in the positions expected for a dark-adapted retina.

FIG. 6. Ommatidium from a day-light eye of *Portunus anceps*. The distal pigment is dispersed proximally toward the basement membrane, while much of the proximal pigment has migrated distally above the basement membrane.

FIG. 7. Ommatidium from a day-dark retina of *Portunus*. The positions of the pigments are the same as in the day-light eye, in spite of the fact that the animals were kept in constant darkness.

FIG. 8. Ommatidium from a night-light eye of *Portunus*. The positions of the retinal pigments are typical for what is expected in an illuminated eye.

FIG. 9. Ommatidium from a night-dark eye of the same species. The distal pigment has migrated distally, while the proximal pigment has moved proximally below the basement membrane.

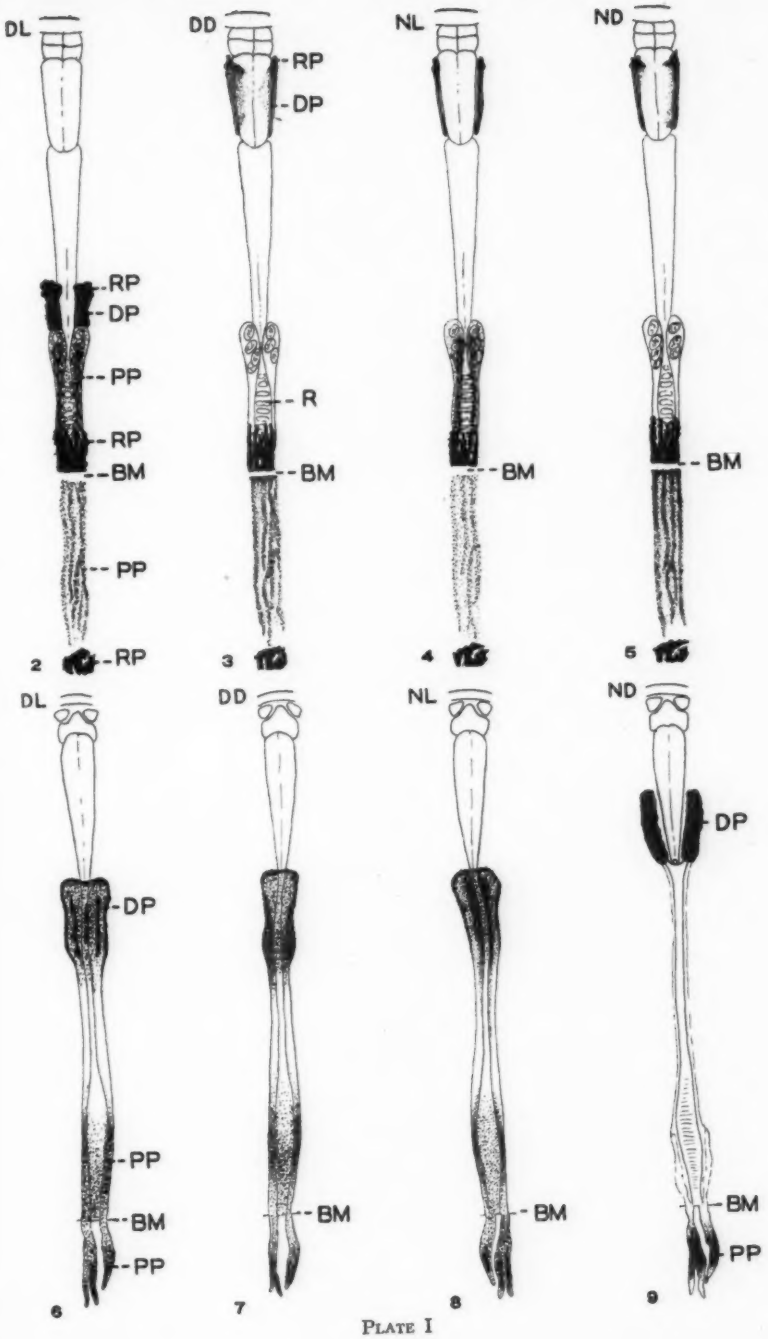
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PLATE I

Day-dark eye

A. The distal pigment is in the position characteristic for the dark-adapted retina, forming a collar around the distal ends of the cones.

B. The proximal pigment has moved entirely below the basement membrane.

Night-light eye

A. The distal pigment, in spite of the fact that the retina has been under constant illumination, is in the position typical for the dark-adapted eye, at the distal ends of the cones.

B. The proximal pigment, however, is in the position found in the usual light-adapted eye, much of it having moved above the basement membrane.

Night-dark eye

A. The distal pigment is in the position characteristic for a dark-adapted retina.

B. The proximal pigment is also in the position found in the dark-adapted eye, having migrated completely below the basement membrane.

Explanation of Plate II

Figs. 10–13 represent retinas of *Eusicyonia* in the four experimental conditions; Figs. 14–17 are photographs of similar retinas of *Trachypeneopsis mobilispinis;* Figs. 18–21 are photographs of the entire stalks of *Portunus anceps.* All the eyes are oriented so that the distal end of the retina is at the left and the proximal end to the right. *D,* distal pigment; *P,* proximal pigment; *R,* reflecting pigment.

Fig. 10. Day-light retina. The distal pigment has migrated proximally, the proximal pigment has moved above the basement membrane and lies directly to the right of the distal pigment, but the reflecting pigment is fixed.

Fig. 11. Day-dark retina. The distal pigment is seen to the left in its distal position, while the proximal pigment has migrated completely below the basement membrane.

Fig. 12. Night-light retina. The distal pigment is in the same position as in the day-dark retina, but the proximal pigment (which has been retouched in this photograph) is in the typical light-adapted position.

Fig. 13. Night-dark retina. The distal and the proximal pigments are in the positions characteristic for a dark-adapted eye.

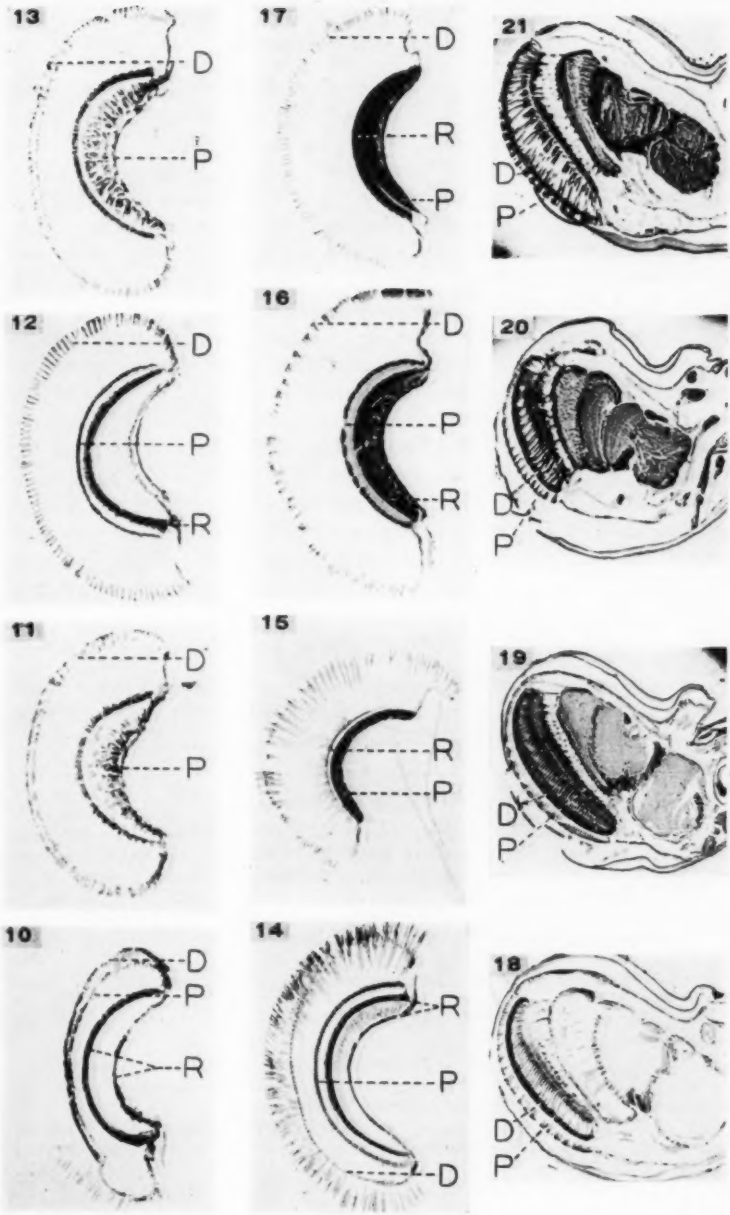
Figs. 14–17 are comparable retinas of *Trachypeneopsis,* arranged in the same sequence as Figs. 10–13 and showing similar movements of the retinal pigments. Fig. 15 shows the very small amount of reflecting pigment in this retina compared with the others of this series.

Fig. 18. Day-light eye of *Portunus.* The distal pigment is the black band to the left. Granules of this pigment are dispersed proximally, while granules of the proximal pigment are dispersed distally towards the left.

Fig. 19. Day-dark eye. The positions of the two pigments are the same as in Fig. 18, in spite of the fact that the animals were maintained in constant darkness.

Fig. 20. Night-light eye. The pigments are in the positions characteristic for a light-adapted retina.

Fig. 21. Night-dark eye. The granules of the distal pigment have migrated distally from the processes in which they had been dispersed, while the proximal pigment granules have moved completely below the basement membrane. The blackness of the processes is due to the stain, and not to any contained pigment.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10
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14
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D
18
D
P

PLATE II
183

Trachypeneopsis mobilispinis

Crustaceans of this species were dredged off sandy bottoms in Castle Harbor and in Bailey's Bay. Eight animals were available for study; three of these were males and the rest females.

A particularly thick exoskeleton and dense pigmentation obscured the eye-stalk so that the positions of the distal retinal cells of whole eyes could not be determined by direct microscopic examination. Study of sectioned retinas, however, revealed a close similarity to the condition found in *Eusicyonia*, the chief difference being in the size relationships of the ommatidial components.

In this species, too, the reflecting pigment apparently undergoes no positional changes. In some specimens this pigment was completely absent, and in one only a trace of it was evident (Fig. 15). The retinal responses to light and to darkness are so similar to those in *Eusicyonia* that the same diagrams of Plate I (Figs. 2–5) serve to illustrate the changes. The distal and the proximal pigments show the usual positional changes in adaptation to light and to darkness. The resemblance to *Eusicyonia* in this respect is heightened by the fact that the persistent diurnal rhythm is found in the distal pigment cells, which, in night-light eyes, are in the position characteristic for a dark-adapted retina.

Brachyurans

Four species of brachyuran crustaceans were studied for the movements of the retinal pigments. Since the responses in all four were found to be identical, they may conveniently be described together.

EXPLANATION OF PLATE III

Figs. 22–25 are retinas of *Parthenope serrata* and Figs. 26–29 represent those of *Calappa flammea*. The retinas are oriented with the distal end at the top of the photograph and the proximal end toward the bottom. *D*, distal pigment; *P*, proximal pigment.

FIG. 22. Day-light retina. The distal pigment is dispersed proximally while the proximal pigment granules have migrated, for the most part, above the basement membrane.

FIG. 23. Day-dark retina. The positions of the two pigments are the same as in the day-light eye, in spite of constant darkness.

FIG. 24. Night-light retina. The pigments are in the positions typical for the light-adapted eye.

FIG. 25. Night-dark retina. The granules of the distal pigment have retreated distally, while those of the proximal pigment have withdrawn completely from the processes to a position below the basement membrane.

FIGS. 26–29 are photographs of retinas of *Calappa* arranged in the same sequence as Figs. 22–25 and showing a similar persistent rhythm in the day-dark eye.

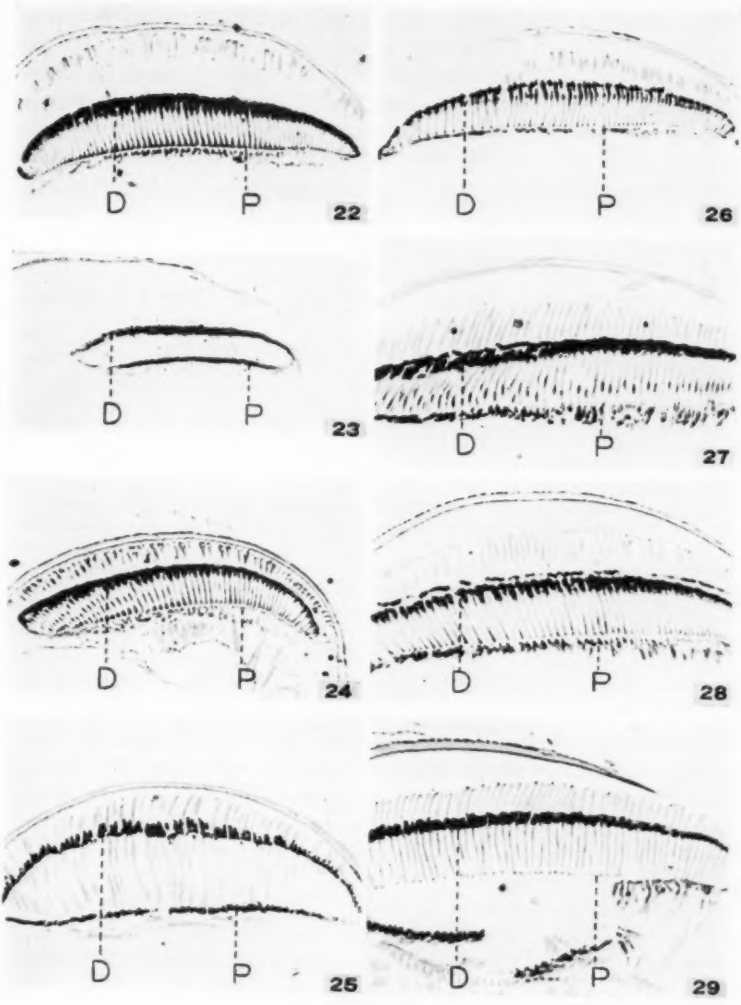

PLATE III

There are two types of black retinal pigments in these animals, a proximal and a distal set of cells; reflecting pigments seem to be completely absent. Diagrammatic illustrations of ommatidia are shown in Figs. 6–9.

The photomechanical changes of the retinal pigments in these brachyurans, although not as marked as those in the macrurans, are still evident:

Day-light eye
- *A.* The distal pigment is dispersed proximally toward the basement membrane.
- *B.* Most of the proximal pigment has migrated above the basement membrane and surrounds the rhabdome.

Day-dark eye
- *A.* The distal pigment remains in the position typical for a light-adapted retina.
- *B.* The proximal pigment also shows the persistent rhythm, remaining in the position found in a light-adapted eye in spite of conditions of constant darkness.

Night-light eye
- *A.* The distal pigment granules are in the same position as in the day-light eye.
- *B.* The proximal pigment is also in the light-adapted position.

Night-dark eye
- *A.* The distal pigment granules have retreated distally from the processes and lie in the cells which are located along the posterior region of the cones.
- *B.* The proximal pigment has moved completely below the basement membrane so that the processes of these cells are entirely free of pigment granules.

Discussion

There is still much ignorance as to the nature of the mechanisms involved in these rhythmic activities. If the retinal pigments were uniform in their periodic responses under constant conditions, the problem as to the mediating agents would be relatively simple. But published accounts of the behavior of these pigments among the different crustaceans reveal perplexing heterogeneities in this respect. Examination of Table I will show the responses reported for various crustaceans.

In the first four species of macrurans, there is a uniformity of response in that the same set of retinal pigment, the distal, shows persistent periodic movements in the same direction under the same conditions of illumination. The next two species of shrimps are unusual in that they show evidence of possessing a double rhythm; not only are distal and reflecting retinal pigments involved in the periodic migration, but the periodicity occurs twice within a 24-hour cycle, once during the daytime when the animals are maintained in darkness, and once at night when the shrimps are kept under constant illumination.

Up to this point, the various responses, although still slightly confusing, are open to interpretation on a hormonal basis as found in the case of *Palæmonetes* (Kleinholz, 1936). The puzzling situations are those where the distal pigments show a persistent rhythm, but the reflecting pigments at the same time undergo only the normal changes in position characteristic for the particular condition of illumination. In *Leander tenuicornis* and in *Latreutes fucorum* the situation is reversed, the reflecting pigments showing the rhythm while the distal pigment cells respond only to differences in light intensity. If endocrine control of these two pigments is universally present in the crustaceans,

TABLE I

Crustacean	Distal	Reflecting	Proximal	Investigator
Macrobrachium olfersii	*NL*	—	—	Welsh (1930*a*)
Macrobrachium acanthurus	*NL*	—	—	Welsh (1930*a*)
Eusicyonia n. sp.	*NL*	*Ab.* or *F*	—	This paper
Trachypeneopsis mobilispinis	*NL*	*Ab.* or *F*	—	This paper
Leander affinis	*DD* and *NL*	*DD* and *NL*	—	Welsh (1935)
Anchistioides antiguensis	*DD* and *NL*	*DD* and *NL*	—	Welsh (1936)
Portunus anceps	*DD*	*Ab.*	*DD*	This paper
Portunus depressifrons	*DD*	*Ab.*	*DD*	This paper
Parthenope serrata	*DD*	*Ab.*	*DD*	This paper
Calappa flammea	*DD*	*Ab.*	*DD*	This paper
Leander tenuicornis	—	*NL*	—	Welsh (1935)
Latreutes fucorum	—	*NL*	*NL*	Welsh (1935)
Cambarus virilis	—	—	*DD*	Bennitt (1932*a*)
Peneopsis goodei	*F*	*F*	*DD*	Welsh (1935)

—, pigment shows normal photomechanical changes but shows no periodicity.
Ab., pigment is absent from the retina.
F, pigment is present, but undergoes no positional changes.
NL, rhythm in a night-light eye, the pigment moving into a typical dark position.
DD, rhythm in a day-dark eye, the pigment moving into the position characteristic for a light-adapted retina.

such differences in response may possibly be due to threshold variations in reactivity to the same hormone, or, there may be two hormones involved, one for the distal pigment and one for the reflecting pigment.

The mechanisms involved in the migration of the retinal pigments in the four brachyurans reported here is less open to analysis chiefly because so little is known about them. These crustaceans are the only ones thus far reported which show a pronounced periodicity in both the distal and in the proximal pigments at the same time. *Cambarus* and *Peneopsis* seem to fall into the same group in that the proximal pigment shows the rhythm.

The mediating agency for the migration of the proximal pigment is not known. In *Palæmonetes* the eye-stalk extracts which affect the distal and the reflecting pigments have no effect on the migration of the proximal pigment. Early workers on the migration of the proximal retinal pigment were of the view that this activity was under nervous control. But as Bennitt (1932*b*) states, the main argument against this belief is that no efferent nerve fibers have been found supplying these cells, their only nervous connection apparently being afferent fibers going to the optic ganglia (Parker, 1895). From the results of his experiments on the interrelation between the eyes of crustaceans (with regard to proximal pigment migration) Bennitt (1932*b*) believes that an endocrine control may be involved.

It is evident from this discussion that the possibility of formulating a general theory for the phenomenon of persisting diurnal rhythm is still remote. The fact that those crustaceans which have been studied lack the uniformity of behavior that is sought by the experimentalist, is probably sufficient proof of the complexity of the mechanism. In our present state of knowledge it can only be hoped that when the retinal pigments of other crustaceans have been studied with regard to hormonal control as in *Palæmonetes*, and the innervation of the proximal pigment cells studied by means of modern neurological techniques, a sufficient amount of information will have been gathered to attempt a general explanation of persistent periodicity in the retinal pigment migration of crustaceans.

Summary

1. Persistence of a diurnal rhythm in the migration of the retinal pigments of several Bermudan crustaceans, in spite of constant conditions of illumination or of darkness, is reported. Two macrurans, a new species of *Eusicyonia* and *Trachypeneopsis mobilispinis*, show the distal retinal pigment in the position characteristic for the dark-adapted eye when the retinas of illuminated specimens are fixed at night.

2. Four species of brachyurans, *Portunus anceps*, *P. depressifrons*, *Parthenope serrata*, and *Calappa flammea*, show the distal and the proximal pigments in the typical light-adapted position when the specimens are kept in the dark-room and are fixed during the day.

3. The retinas of some of the macrurans showed deficiencies or absence of the reflecting pigment. This is thought to be due to differences in metabolism rather than to fixation artifacts.

4. Hormones are considered as the possible mediating agency involved in the phenomenon of persisting diurnal retinal rhythms.

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BACTERIA AND THE PHOSPHORUS CYCLE IN THE SEA[1]

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Marine bacteria, growing and multiplying rapidly under favorable conditions, are potential accumulators of the sea's labile organic matter. Lohmann (1911) has estimated that they may bind as cell substance each year from six to three hundred times the annual production of slower growing nannoplankton feeding upon them.

It is surprising, then, to discover, as Fischer (1894) and many others since have pointed out, that the bacterial population of the sea and large fresh-water lakes is very low—rarely exceeding a viable count of a hundred at the surface and extending through a lesser range in the depths (Birge and Juday, 1922; Reuszer, 1933). Lohmann found the mass of bacterial cells in sea water to represent an insignificant fraction of the nannoplankton; and it may be calculated, making generous allowance for non-viable cells, that the amount of phosphate, nitrate, or other nutrient substance bound by bacteria is immeasurably small. Thus, a million cells per cubic centimeter, at least a thousand times the likely bacterial population, would be equivalent to less than 3 mgm. PO_4 per cubic meter (Buchanan and Fulmer, 1933, pp. 69–71). Bacterial cell substance is notoriously rich in phosphorus, but it is apparent from their small numbers, that bacteria do not compete effectively for the sea's limited store of this element. Do these small numbers also indicate a low limit to their activity in the breakdown of debris and the regeneration of inorganic nutrients?

If Fischer's observations regarding the short life of marine bacteria are valid (he estimated their average viable span to be in the order of a half hour), then Lohmann's emphasis on their rôle as converters of organized material rather than accumulators gives a sound picture of their relations in marine economy. Rate of growth, length of the viable period, and rapidity of autolysis after death become critical factors in determining their value as regenerating agents.

Several experiments make it seem plausible that short-lived bacteria are active in phosphate regeneration, and offer confirmation for the smaller, inter-seasonal phosphorus cycles indicated in the investigations

[1] Contribution No. 133 from the Woods Hole Oceanographic Institution. Contribution from the Bermuda Biological Station for Research, Inc.

of Harvey and co-workers (1935) on plankton production, and remarked by Seiwell (1935). When specimens of sea water are stored in bottles at room temperature the bacterial population therein rises in two or three days to a level much higher, from a thousand to a hundred thousand times, than that of natural waters. This sudden increase is followed by a precipitous drop in viable numbers to a persistent low count (Waksman and Renn, 1936). That such unrestricted development of bacteria may withdraw appreciable quantities of phosphate from

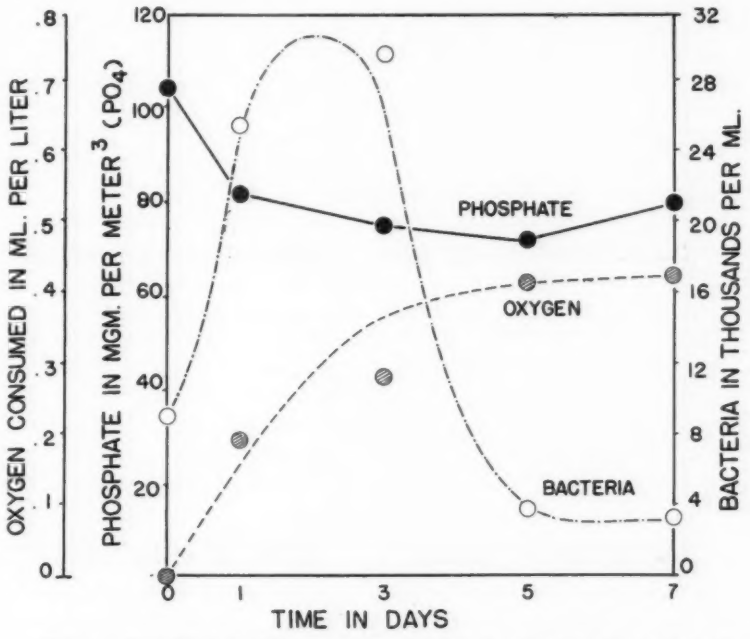

FIG. 1. Showing assimilation and regeneration of phosphate by bacteria in stored samples of sea water from Woods Hole Harbor—July 10, 1935. Phosphates in triplicate, other determinations in duplicate.

sea water is shown by the decreasing concentrations in unpreserved specimens taken for analysis during the first two or three days of storage.

The first experiment was designed to relate these two behaviors—increase in bacterial numbers and assimilation of phosphate. Water from the mouth of Woods Hole Harbor was enriched with K_2HPO_4 to bring the phosphate content to about 100 mgm. per cubic meter, filtered

through quartz sand, and dispensed into sterile, standard, oxygen demand bottles. These were stored in the dark at room temperature for varying lengths of time after which phosphate, bacterial number, and dissolved oxygen determinations were made. Figure 1, incorporating the results, clearly shows the rapid decrease in phosphate during the first three days of storage as noted before. Simultaneously the bacterial numbers and oxygen consumption reach their maximum. After three days the viable bacteria fall to a low level, and the oxygen uptake

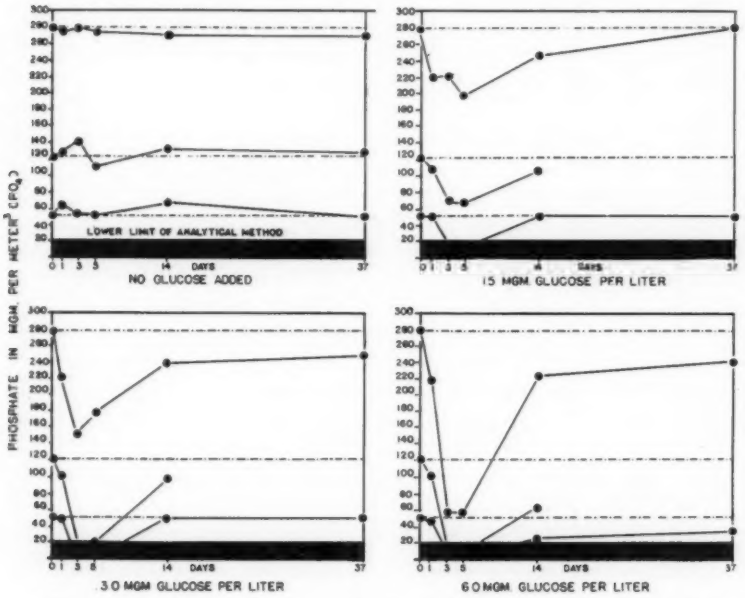
PHOSPHATE IN MGM. PER METER³ (PO₄)
LOWER LIMIT OF ANALYTICAL METHOD
NO GLUCOSE ADDED
15 MGM. GLUCOSE PER LITER
30 MGM. GLUCOSE PER LITER
60 MGM. GLUCOSE PER LITER
DAYS

FIG. 2. Showing assimilation and regeneration of phosphate in stored sea water from off Bermuda, January, 1936. All determinations in triplicate.

slackens off. On the seventh day there are signs of phosphate regeneration.

There is, then, an interval of two or three days in which 20–25 mgm. of phosphate per cubic meter is bound as non-viable bacterial cells. (The count of viable cells has qualitative value only, indicating the phase of growth rather than actual numbers. Assimilated phosphate is held by a quantity of bacterial cells that is roughly proportional to the organic carbon oxidized during rapid growth—in this case an equivalent of 0.75 mgm. glucose per liter.) It has been pointed out that the length

of time that phosphorus may be held by bacterial cells synthesized during decomposition of organic phosphorus compounds is significant in estimating their activity in phosphate regeneration. If this is long, then the process is retarded, but if, as this preliminary experiment suggests, the cells lyse within a short time, the turnover through their agency may be rapid.

In January, 1936, there was an opportunity to study the complete cycle of bacterial assimilation and regeneration under particularly favorable conditions, using the waters about Bermuda and the facilities at the Bermuda Station for Biological Research. The waters in this region are characteristically poor in phosphate, organic phosphorus, and available carbon—components that had proven troublesome in attempts to extend the experiments in Woods Hole. (A number of determinations showed the total phosphorus in shallow bays between St. Georges and Shore Hills to be less than 40 mgm. PO_4 per cubic meter.)

Sea water, taken from the surface about three miles southeast of Castle Roads, was filtered through quartz sand (imported), and enriched with varying amounts of phosphate and glucose, and a uniform excess of nitrate and iron. This was dispensed into a series of three-liter bottles and stored in the dark at room temperatures of 19° to 21° C. At intervals specimens were taken for phosphate analyses.

Results of the periodic inorganic phosphate analyses on the prepared, stored water are given in Fig. 2. Phosphate assimilation is complete in about five days, by which time it may be assumed bacterial growth has passed its maximum. Regeneration follows almost at once —rapid at first, and then leveling at fourteen days toward completion. Most of the phosphate is regenerated within this time, but a fraction, ranging from an eighth to a third, and greatest where relatively large quantities of glucose have been added, is still bound at the end of thirty-seven days when the experiment was terminated. It seems clear that bacterial cells do not bind phosphorus for more than a few days under storage conditions.

If autolyzing bacterial cells liberate phosphorus as phosphate there always exists the possibility that diatoms and other phytoplankton may similarly bring about direct regeneration. Two attempts to demonstrate direct regeneration gave negative results, but it will be recognized from the methods followed that the mechanism is by no means disproved. Ten-day cultures of *Nitzchia* grown in synthetic media were filtered and washed free of inorganic phosphate on a Seitz filter. The cells were suspended in 200 ml. of distilled water and washed on a second filter with 800 ml. of sea water, the lysate passing into a sterile aspirator

bottle fitted with tubes for periodic sampling. Phosphate analyses at intervals of 14, 20, and 48 hours indicated no increase whatever in inorganic phosphate content.

A modification was arranged in which cultures of *Nitzchia* were washed on the Seitz filter, removed with a thin layer of the filter pad, lysed in distilled water for two hours, suspended in ten volumes of sea water, and allowed to stand. After three and eight hours, respectively, specimens were centrifuged and analyses made. These yielded no measurable amounts of phosphate.

Discussion and Summary

Bacteria, despite their potentialities as rapidly multiplying organisms, do not compete with higher phytoplankton for phosphate. Many factors may be suggested to account for their low numbers, but short life and brief viability period undoubtedly play a part. Under storage conditions where their rapid growth is favored they assimilate significant quantities of phosphorus, but this is quickly regenerated following their death. This suggests that the numbers of viable cells in natural water is not a complete index of bacterial activity, and that the efficiency of the organisms as agents of regeneration is inversely proportional to their life span.

The relations of the rapid growth phase, short period of maximum viable population, and the declining phase are not unique in marine bacterial cultures but are characteristic of bacterial populations in general (Buchanan and Fulmer, 1933, pp. 69–71). The curve representing them is the resultant of many growth and death curves over much shorter intervals, so that the number of cells actually produced during three, five, seven or fourteen days is indeterminately greater than the population at any one time.

Many other mechanisms for phosphate regeneration may be active in the sea. These experiments demonstrate one possible system, involving the agency of bacteria. They also offer an explanation for the insignificant amounts of bacterial cell substance in the nannoplankton.

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INDUCTION OF ENDOMIXIS IN PARAMECIUM AURELIA

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Attempts to induce endomixis in *Paramecium aurelia* have in the past led to conflicting results. Jollos (1916) and Young (1917) reported successful inductions; but Woodruff (1917) found that the time of occurrence of endomixis was but slightly modifiable by external conditions. Erdmann (1920) shared Woodruff's views on the inner determination of endomixis and rejected the results of Jollos and Young as unsound. The present paper, in general agreement with Jollos, reports a reliable method of inducing endomixis and sets forth the quantitative differences in response to this method at different periods of the interendomictic interval and in different stocks of the species.

The procedure employed for inducing endomixis was that which has been described in earlier papers (Sonneborn, 1936). The organisms were under culture in isolation lines under uniform conditions. To induce endomixis, the surplus animals available after the daily transfer of the lines to fresh culture fluid were collected into 10 drops (approximately 625 cu. mm.) of fresh culture medium in a Columbia dish, and were kept without further change at 31° ± 1° C. By culture in this manner endomixis was induced, as hereafter described.

Two stocks of *Paramecium aurelia* were studied with relation to the induction of endomixis: these were the stock R, dealt with in earlier papers by the author and associates, and Woodruff's long-lived stock, here designated W. At various times over a period of three years, 11 groups of lines of stock R and 8 groups of stock W were investigated.

Of the 19 groups studied, 18 were started with one or a few animals in the climax of endomixis, while one was started with 24 exconjugants. In each group begun with animals in endomixis, the few original animals were soon expanded into usually 12 isolation lines. In all groups, one or more individuals from each isolation line were stained daily, to determine the occurrence of endomixis. Any line that was found in endomixis was discarded, in order that the interval of time since the previous endomixis should be the same for all the individuals placed in any one of the small mass cultures.

The mass cultures derived as above described from these isolation lines were observed at intervals not exceeding 12 hours, to determine

whether conjugation was in progress. Also, a random sample of 20 to 50 individuals of each culture was stained usually once a day to determine whether endomixis was in progress.

Stock R

As set forth in an earlier paper (Sonneborn, 1936), mass cultures of stock R set up soon after endomixis or conjugation, in the way above described, soon pass into conjugation, so that such cultures are unavailable for the study of the induction of endomixis. But when the interval from the preceding nuclear reorganization (endomixis or conjugation) is greater, conjugation does not occur, but some of the individuals pass into endomixis. The percentage of those thus undergoing endomixis was accurately determined, and compared with the percentage occurring in the isolation cultures (still in progress) from which they were derived. In this way the effect of the mass culture conditions in inducing endomixis is numerically expressed.

TABLE I

Stock R. The percentages of daily isolation lines at 27.5° C. in endomixis on successive days of the interendomictic interval, compared with the percentages of animals in endomixis in mass cultures at 31° C. on corresponding days. In each case, the mass cultures had been derived from the isolation lines on the preceding day.

Day since initial endomixis	Percentage of endomixis	
	In isolation lines at 27.5° C.	In mass cultures at 31° C.
9	0	0
10	0	0
11	0	0
12	8.3	37.6
13	0	58.7
14	0	39.7
15	16.7	53.0
16	42.9	93.4
17	0	98.3
18	50.0	97.5
19	57.1	97.5
20	0	100
21	66.7	97.0
22	0	100
23	0	100
24	100	100

Typical results are given in Table I. From the twelfth until the twenty-fourth day after the previous endomixis, the percentage of individuals in endomixis in the mass cultures is always much greater than that in the isolation cultures (until by the twenty-fourth day the last isolation line has passed into endomixis, bringing the comparison to a

close). With increasing time since the preceding endomixis, the percentage of individuals in which endomixis was induced quickly rose from 37.6 per cent on the twelfth day to 98.3 per cent on the seventeenth day, after which it remained at or near 100 per cent. Similar results, differing only in details, were obtained with the other groups of stock R, including the group begun with ex-conjugants.

These results thus confirm the reports of Jollos (1916). Endomixis is induced by the treatment set forth and, within the range of intervals examined, it is induced less readily the shorter the interval since the preceding endomixis. This relation to the time since a previous endomixis is also shown in other ways. (1) It took a longer period in mass culture to induce endomixis in the earlier mass cultures derived from given isolation lines than in the later ones. Thus the mass cultures set up on the seventh to tenth days of a certain series yielded no endomictic animals until these cultures were two days old, whereas in the later cultures of the series endomixis was induced in one day. (2) The production of large percentages of endomixis in the early cultures required a type of treatment that was unnecessary in the later cultures. Thus, a culture set up on the tenth day after endomixis yielded no more than 10 per cent endomixis on any of the four days it was examined, but a subculture from it set up on the third day and provided with fresh culture fluid gave 44.4 per cent endomixis on the following day. Similar results were obtained with other cultures: a culture set up on the eleventh day after endomixis gave but 5.4 per cent endomixis on the second day; it was then divided into two parts one of which was provided with 5 drops of fresh culture fluid, the other not. On the next day, the former had 69.2 per cent endomixis, the latter 5.8 per cent. It appeared that the percentage of endomixis in these early cultures reached its maximum at about the time when the food supply was giving out and that further increase in the percentage of endomixis could be obtained only by supplying additional food.

The relations observed were usually of the type just set forth, but very rarely exceptional results were obtained. In lines descended from one endomictic individual, mass cultures set up on the first, third, and fifth days after endomixis yielded at once another endomixis instead of conjugation, as would normally have been expected at this period. These lines also went into endomixis after 5 to 8 days in the isolation cultures. It is perhaps significant that in this case the initial nuclear reorganization was highly abnormal: on the day following the stage of complete macronuclear fragmentation there were neither numerous small fragments nor pale anlagen of the new macronucleus, such as normally occur; instead, several large, aggregated spheres of chromatin

were present. Thereafter, normal-appearing macronuclei were characteristic of this line. Similar abnormal reorganizations have on several occasions been followed by extraordinarily short intervals, and appear to be the basis of the unusually quick recurrence of endomixis in these cases.

Stock W [1]

In stock W, Sonneborn and Cohen (1936) have shown that mass cultures set up in the way here set forth do not yield conjugants, and examination at not more than 12-hour intervals showed that conjugation did not occur in the present cultures. This made it possible to determine from the start the percentages of individuals in endomixis.

In Table II are given, for a typical group of lines of stock W, the percentages of individuals in endomixis in the mass cultures set up on successive days of the interendomictic interval and kept for various periods of time at 31° C. The table is to be read as follows: the culture set up on the second day yielded no endomixis; the culture set up on the third day yielded 8.6 per cent endomixis after five days at 31° C., i.e., $5 + 3 = 8$ days after the initial endomixis and so on. Table II shows that endomixis was regularly induced every day beyond the seventh after the initial endomixis. In the isolation lines endomixis did not occur until the thirtieth day.

In some of the other groups, the earliest induction of endomixis occurred sooner than in the typical group. In two groups endomixis was induced four days after the initial endomixis and in one group, three days. Usually, however, at first only small percentages of endomixis could be induced after 5 days of treatment. Later, comparable small percentages were induced in four days of treatment. Still later, treatments of four days yielded nearly 100 per cent endomixis; then 100 per cent endomixis was obtained in three days and finally in two days. In contrast to stock R, which yielded 100 per cent endomixis in one day in the later cultures of a series, stock W never yielded 100 per cent endomixis in less than two days of treatment.

In stock W, as in stock R, the percentage of induced endomixis increased with the time since the preceding endomixis. This is shown in Table II and also by a comparison of the percentages of endomixis induced on the same day in the cultures of several groups differing in time since the last endomixis. Thus, the mass cultures set up on a certain day yielded after two days 100 per cent in a group 24 days past endomixis, 97.4 per cent in a group 19 days past endomixis, and 8.2 per cent in a group 4 days past endomixis. In two-day-old cultures

[1] In the work with stock W, the author was greatly assisted by Dr. B. M. Cohen.

set up on another day there were 100 per cent in a group 23 days past endomixis, 30.8 per cent in a group 18 days past endomixis, and 0 per cent in a group 13 days past endomixis.

TABLE II

Stock W. The percentage of individuals in endomixis in mass cultures set up daily from a group of isolation lines. Cultures set up from the first to the twenty-eighth day after the initial endomixis and kept at 31° C. for 1 to 5 days. During the entire period there was no endomixis in the isolation lines from which the mass cultures were derived.

Days from endomixis till mass cultures set up	Percentage of endomixis in mass cultures				
	Number of days since culture set up				
	1	2	3	4	5
2	0	0	0	0	—
3	—	—	—	—	8.6
4	—	—	—	—	29.7
5	—	—	—	6.5	—
6	—	—	—	—	26.8
7	—	—	—	83.9	—
8	—	—	65.6	97.1	—
9	—	32.3	69.1	97.8	—
10	0	36.7	100	—	—
11	0	96.9	—	—	—
12	0	56.0	100	—	—
13	0	60.6	100	—	—
14	0	100	—	—	—
15	3.3	100	—	—	—
16	0	96.3	—	—	—
17	20.0	97.4	—	—	—
18	0	—	100	—	—
19	—	92.9	—	—	—
20	0	100	—	—	—
21	—	100	—	—	—
22	10.5	100	—	—	—
23	—	85.3	—	—	—
24	—	93.3	—	—	—
25	—	48.4	47.6	48.5	50.0
26	—	23.5	77.8	—	—
27	—	88.9	—	—	—
28	—	96.9	—	—	—

On the other hand, factors other than the time since the last nuclear reorganization influence the percentage of endomixis induced. There were many instances in which the percentage of endomixis was less at a later period after endomixis than earlier. For example, in Table II, there was 96.9 per cent endomixis after two days treatment of the culture set up on the eleventh day after endomixis, and only 56.0 per

cent in the culture set up a day later. Such irregularities were probably due to differences in the environmental conditions at different times, as indicated by the fact that the same departure from the general trend was usually shown by all the cultures set up on a particular day. An example of the effect of one such environmental change was provided by the accidental rise of temperature to 35° C. in the box ordinarily kept at 31° C. In four groups of cultures exposed to this high temperature, the percentages of endomixis were 25.7 per cent, 32.9 per cent, 36.0 per cent, and 0 per cent, as compared with 79.8 per cent, 75.1 per cent, 96.7 per cent, and 43.9 per cent, respectively, for these same four groups on the two preceding days at 31° C. Thus, rise of temperature from 31° to 35° reduced the percentage of endomixis induced; although, as earlier shown, rise from 27.5° to 31° greatly increased the percentage of endomixis. There are doubtless other as yet unanalysed factors influencing the induction of endomixis.

Summary

In both stocks R and W of *P. aurelia,* endomixis can be induced by placing at 31° C. small mass cultures containing the surplus animals from isolation lines. In stock R, induction cultures set up soon after endomixis yielded conjugants, so that the induction of endomixis could not be studied quantitatively in these. Later cultures, however, showed increasing percentages of induction until 100 per cent was obtained after one day at 31° C. Earlier cultures which gave but low percentages of endomixis with this treatment could be induced to give larger percentages by subculturing or adding some fresh culture medium to the original induction culture. In a few exceptional cases, abnormal reorganization of the nuclear apparatus was soon followed by a normal endomictic reorganization in isolation lines, and by the induction of endomixis instead of conjugation in the induction cultures set up immediately after the abnormal reorganization.

In stock W, the absence of conjugation made it possible to examine the percentages of endomixis induced at all periods of the interendomictic interval. During the first few days after an endomixis, it required prolonged treatment to induce even a low percentage of endomixis, but as the time since the last endomixis increased, higher and higher proportions of endomixis were induced with shorter and shorter treatments, until 100 per cent endomixis could be induced in two days. Irregularities in this progression are probably due to environmental differences in treatment from day to day. Rise of temperature above 31° C. was shown to reduce the percentage of endomixis induced. Other conditions also probably play a similar rôle.

Stocks R and W differed in two respects in regard to their reaction to the conditions favoring the occurrence of endomixis. (1) In stock W, endomixis could regularly be induced 7 days after a preceding endomixis, and in some cases, as early as the third day; but in stock R, the typical response at this period was conjugation, not endomixis. (2) Late in the series of induction cultures, 100 per cent endomixis could be induced with a treatment of one day in stock R, but treatment of two days was required in stock W.

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SOME EFFECTS OF DIET ON THE GASTRIC EPITHELIAL CELLS OF THE GRASSHOPPER, MELANOPLUS DIFFERENTIALIS THOMAS[1]

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Introduction

The effects of variation in diet on various tissues has been investigated in many species of vertebrates (d'Ancona, 1927; Truszkowski, 1927 and 1928; Tirelli, 1928; Smith, 1929; Kremer, 1932). The aim of this study is to determine the effect of diet on the tissues of the grasshopper. A preliminary study disclosed that the epithelial cells of the anterior lobes of the gastric cæca are most favorable for this study.

The six gastric cæca in this species are arranged in a circle around the cephalic end of the mid gut as bilobed diverticula. which are thicker in the anterior lobe than in the posterior. The gastric epithelium is thrown into 6 large and 6 to 8 smaller, inwardly projecting longitudinal folds or villi which are the most concerned with secretion (Woodruff, 1933).[2] Between these villi are longitudinal crypts corresponding in number and arrangement with the villi. This epithelium is composed of functional secretory cells which are elongate and columnar, and are provided distally with a thick striated border of cilia-like filaments. Scattered irregularly through the epithelium are the usual nidi of regenerative cells for replacement.

Materials and Methods

The grasshoppers were reared on the leaves of barley or wheat, alone or in combination, and on oat leaves alone. It had been previously determined from growth data (Hodge, 1933) that any of the above diets except oat leaves are satisfactory. This study also shows that the various satisfactory diets do not differ materially from each other in their effect on the cells.

[1] Contribution No. 8 from the Biological Laboratories of Temple University.

[2] "'Villus,' as used by Sanford (1918) is preferable to 'fold,' as applied to the typical elevations in the gastric cæca, inasmuch as these elevations are permanent structures. Even 'villus' is not entirely satisfactory for the reason that the structure is so extensive in a longitudinal direction," Woodruff (1933), p. 56.

Alimentary tracts from the grasshoppers so reared were fixed in B3 (Carother's modified Bouin), sectioned at 12 microns, and stained with hæmatoxylin and eosin. Eleven cæca of satisfactorily fed grasshoppers and nineteen of those fed on oat leaves were studied. The diameter of the cæcum was measured in four directions and an average taken. The number of cells per section, the number of secretory inclusions, and the number of mitoses visible in the nidi were counted.

One hundred cells located on the villi, 100 from the crypts of the satisfactory diet, 147 from the villi and 148 from the crypts on the oat diet were studied. Usually 5 and never more than 10 cells from any one section were selected. The length and width of the cell and nucleus and the length of the filaments of the striated border were measured.

The volume of the cells was calculated as a rectangular prism. In areas where some cells were sectioned transversely, the cells varied in number of sides from 3 to 6. The majority had 4 sides and the remainder varied in approximately equal numbers on either side of that figure. The volume of the nuclei was calculated as an oblate spheroid.

Results

Cæcum Diameter

The gross morphology of the cæca and the pattern of distribution of the histological elements were not affected by diet. As previously determined (Hodge, 1933), growth on the oat diet is slow, irregular, and retarded, and mortality is high. Only one adult was available for this study. It showed an average diameter through the thickest part of the cæcum of 1,173 microns. Others in the last instar ranged from 667 to 992 microns, with the average 791. The average for all on the oat diet including the adult was 890 microns. On the satisfactory diet all were adults. On this diet the cæcal diameter ranged from 670 to 1,169 microns, with an average of 952. Thus the adults on the satisfactory diet showed an average cæcal diameter 1.07 times that of the 18 last instar and one adult individual on the oat diet; or the oat diet, 93.5 per cent that of the satisfactory. In view of the wide individual variation, this figure is hardly significant. Gross morphology is not affected by this difference in diet.

Number of Cells

The number of cells per section on the satisfactory diet ranged from 365 to 775 (average 552.7). The number on the oat diet, from 340 to 648 (average 489.0). Thus the average number of cells per section

for the satisfactory diet was 1.13 times that for the oat diet; or the oat diet, 88.5 per cent that of the satisfactory.

A comparison of the number of cells per unit area is difficult. No two cæca are exactly the same shape nor are they provided with villi and crypts to exactly the same extent. However, a ratio between the average diameter of the cæcum and the number of cells yields a rough comparative figure. For the satisfactory diet this ratio ranges from 1.34 to 2.38 for the various cæca, with an average of 1.79. For the oat diet the range is 1.36 to 2.02 with the average 1.82. Thus the average ratio for the satisfactory diet is 98.4 per cent that of the oat diet; for the oat diet, 1.02 times that for the satisfactory.

TABLE I

	Inclusions per Section		Cells per Section	Percentage of Cells with Inclusions			Ratio, Satisfactory: Oat Diet			Ratio, Oat: Satisfactory Diet		
	Granules	Vacuoles		Granules	Vacuoles	Either	Granules	Vacuoles	Both	Granules	Vacuoles	Both
Satisfactory diet												
Minimum	12	29										
Average	35.8	43.5	552.7	6.5	7.9	14.3	1.35	2.93	1.86			
Maximum	83	78										
Oat diet												
Minimum	12	8										
Average	23.5	13.2	489.0	4.8	2.7	7.7				.738	.342	.538
Maximum	30	30										

Rate of Replacement of Cells

Characteristic of insect mid-gut epithelium are the nidi from which new secreting cells for replacement proliferate by mitosis. The number of mitoses visible at any one time gives an indication of the rate of replacement. The satisfactory diet showed 4 to 8 mitoses per section, with an average of 6.4. The oat diet showed 8 to 14 with an average of 11.6. Thus the rate of replacement for the satisfactory diet was 55.2 per cent that of the oat diet; or the oat diet, 1.8 times that for the satisfactory.

Secretory Products

By the technique used here the secreting epithelial cells show two types of inclusions: darkly-staining granules and clear vacuoles. They

originate just distad to the tip of the nucleus and are progressively larger as they are found successively nearer the distal end of the cell. I interpret them as some of the gastric secretory products (Hodge, 1936). The numbers of these inclusions are given in Table I. Thus the satisfactory diet showed 1.35 times as many granules, 2.93 times as many vacuoles, or 1.86 times as many inclusions, as the oat diet. The oat diet showed 74 per cent, 34 per cent, and 54 per cent, respectively, as many as the satisfactory.

Dimensions of Cells

The average values in microns and cubic microns for the dimensions measured: length, width and volume of cell and nucleus, and width of striated border, are tabulated below. The average for all the cells on the satisfactory diet differed only very slightly from that for all the cells on the oat diet. The greatest variation is seen in nuclear volume and the length of the filaments of the striated border. In general the cells and their nuclei on the satisfactory diet are slightly longer and more slender than those on the oat diet and the filaments of the striated border are shorter:

Diet	Cell Length	Cell Width	Cell Volume	Nuclear Length	Nuclear Width	Nuclear Volume	Striated Border	Nucleo-plasmic Ratio
Satisfactory...	61.6	12.2	9484.7	16.9	8.1	580.7	5.0	1 : 16.3
Oat.....	58.3	12.6	9450.7	16.3	8.6	663.0	5.6	1 : 14.3

In view of the great variation between minimum and maximum values for these various dimensions (see the more extended data in Table II) these differences are hardly significant. More striking differences are observed in comparing the dimensions of cells located on the villus and crypt areas respectively. On the satisfactory diet the cells vary greatly in relation to their location on these two areas. In general on the villi the cells are shorter and wider, the striated borders narrower, and the nuclei more slender than in the crypts. On the oat diet the cells of the two areas are more similar. This may be summarised as in Table II.

Discussion

The diet of oat leaves alone is undoubtedly unsatisfactory for this species. This has been shown by rate of growth (Hodge, 1933) and

by abnormalities in cytology of the germ cells (C. E. McClung, personal communication, unpublished).

This study shows that the rate of replacement of the cæcal epithelial cells on the oat diet is almost double that on the satisfactory. Replacement is more or less continuous in insect mid-gut epithelium. Appar-

TABLE II

	Satisfactory Diet			Oat Diet		
	Villus	Crypt	Ratio Villus : Crypt	Villus	Crypt	Ratio Villus : Crypt
Cell length						
Minimum	29	44	.8 : 1	40	37	.9 : 1
Average	55.9	67.0	.8 : 1	56.7	59.8	.9 : 1
Maximum	95	99		88.1	84	
Cell width						
Minimum	6	8		6	6	
Average	12.4	12.0	1.0 : 1	13.2	12.0	1.1 : 1
Maximum	18	17		21	18	
Cell volume						
Minimum	2245	3213		2069	2829	
Average	8891.4	10077.9	.9 : 1	10012.7	8902.3	1.1 : 1
Maximum	26741	22289		23286	23621	
Nuclear length						
Minimum	11	11		9	11	
Average	16.9	16.9	1.0 : 1	16.6	16.0	1.0 : 1
Maximum	23	26		26	22	
Nuclear width						
Minimum	5	6		5	5	
Average	6.7	9.0	.7 : 1	8.1	9.1	.9 : 1
Maximum	11	14		13	16	
Nuclear volume						
Minimum	171.5	367.6		111.9	252.2	
Average	364.4	796.9	.5 : 1	561.9	762.0	.7 : 1
Maximum	838.3	2060.5		1372.2	1658.9	
Striated border						
Minimum	2	3		3	4	
Average	3.7	6.3	.6 : 1	4.9	6.4	.8 : 1
Maximum	6	12		8	11	
Nucleo-plasmic ratio	1 : 24.4	1 : 12.6	.5 : 1	1 : 17.8	1 : 11.7	.7 : 1

ently it is necessitated by the exhaustion of holocrine secretory cells, the gradual wearing out of merocrine cells, or other factors.

On the oat diet the number of the large holocrine type of secretory inclusions demonstrated by the technique used here is less than on the satisfactory diet. If the greater replacement results from overstrenuous secretion, it must be of the merocrine type demonstrated by Woodruff (1933) and others.

On the satisfactory diet the more actively secreting cells of the villi are conspicuously smaller than the crypt cells. From this one is led to the generalization that among these cells the smaller type is the secreting type. On the oat diet the cells, though shorter in length of nucleus and cell body, are yet for all other dimensions as large or larger than those from the satisfactory diet, and in each case show less difference between the villus and crypt areas.

On the whole, therefore, the villus and crypt cells on the oat diet are more similar morphologically than the villus and crypt cells on the satisfactory diet. Furthermore, their general morphology approaches more nearly to that of the larger, less actively secreting crypt cells. If morphology be taken as a criterion of secretory function the oat cells are not secreting more strenuously, but less.

Other factors that suggest themselves as reasons for wear and replacement are the results of toxic influences, and inanition. Toxic materials resulting from the digestion of the oat leaves have not been demonstrated directly, but the high mortality among grasshoppers on the oat diet (Hodge, 1933) may be due to toxicity. The fact that the cells on the oat diet are provided with longer filaments of the striated border is also suggestive. Longer filaments might conceivably have a distinct protective function, especially since these can be demonstrated to be covered with a mucous layer in the living condition (Hodge, 1936).

Inanition in other material (Miller, 1927, on rats; Sun, 1927, on mice) produces loss of digestive epithelium. This desquamation, in animals so dissimilar on the part of a tissue not normally so adapted, cannot be too closely analogized to the replacement in the grasshopper. But it may well be that the greater replacement on the oat diet may be due to at least two factors—toxic effect of the diet and a partial inanition.

The two types of large secretory inclusions demonstrated by the technique used here—namely, granules and vacuoles—are shown in noticeably larger number on the satisfactory diet than on the oat diet. One and one-third times as many granules, and almost three times as many vacuoles were counted on the satisfactory diet as on the oat diet.

The nature and function of these two types of inclusions are as yet uncertain. If they represent two distinct types of enzyme material, it would seem that the oat diet hinders the formation of the vacuolar type more than that of the granular type. However, in each case either some dietary component necessary for their elaboration is relatively deficient in the nutrient materials the grasshopper can obtain from the oat leaves, or an injurious material either directly or indirectly obtained

from the same source inhibits their elaboration. This difference may be the result of a generalized partial inanition, but in other material inanition leads to vacuolization rather than the reverse (d'Ancona, 1927; Tirelli, 1928; Miller, 1927).

The difference in dimensions between the crypt and villus cells on the satisfactory diet is understandable from the fact that the villi are the more actively secretory (Woodruff, 1933). In the case of each dimension studied except cell width and nuclear length the cells of the villi give much the smaller figure. The corresponding dimensions for the oat diet, while in most cases smaller for the villus than for the crypt cells, do not show such wide divergence. For some dimensions, in fact, the average value for the villus cells is greater than for the crypt cells. Thus on the oat diet the villus cells in size are more like the crypt cells which secrete less; and from this morphological criterion are less differentiated for the normal function of villus cells,—namely, secretion.

The slightly higher nucleo-plasmic ratio on the oat diet suggests an inanition. Such an increased nucleo-plasmic ratio has been reported in other material during inanition (d'Ancona, 1927, on eels; Kremer, 1932, on frogs; Truszkowski, 1927, on dogs, and 1928, on frogs).

A comparison of the relation of the nucleo-plasmic ratio in the crypt areas with that in the villi shows that on the satisfactory diet the ratio in the crypts is twice that in the villi, while on the oat diet it is only 1.5 times as great. This suggests again the conclusion borne out by the dimensions of the cells, that there is less difference between the types of activities of the cells in these two areas respectively on the oat diet than on the satisfactory.

The difference in length of the filaments of the striated border is less readily explicable. The exact purpose of the striated border is not yet well understood. The secretory products are discharged through this border. This is effected by total rupture of the border or by a temporary separation of the filaments into clumps or pencils, depending on the nature of the secretory operation at the moment. The shortness of the filaments on the actively secreting villi on the satisfactory diet would seem to be an adaptation to this function, to permit easy passage of the secretion.

If the striated border subserves any active part in secretion it would seem from the length on villus and crypt respectively on the satisfactory diet that shortness of filaments is in some way correlated with active secretion. From this point of view the greater length of the filaments on the oat diet, correlated with the lesser number of inclusions, points to decreased secretion on this diet.

The fact that on the oat diet the filaments on the villi are more nearly the length of those in the crypts suggests again a partial relinquishment of the active secretory function of the villus. This area seems to have assumed a more generalized condition more nearly resembling the crypts.

If the striated border serves more particularly as a protective surface, the increased length of the filaments on the oat diet would point to a greater need for such protection. Some product of injurious nature from the wholly or partially digested oat leaves is indicated. The shorter filaments shown on the satisfactory diet would indicate that such products are not liberated in the digestion of the wheat or other satisfactory food plants.

At least three factors are indicated by this study as contributing to the various effects of the unsatisfactory oat diet. Inanition is evidenced by the greater rate of replacement and nucleo-plasmic ratio on the oat diet. The retarded growth observed in rearing the animals (Hodge, 1933) is further evidence of this. Injury by or toxicity of some product of digestion or component of the oat leaves is also suggested by the increased rate of replacement. Perhaps the longer filaments of the striated border are also a response to some such factor, as a protective adaptation. The mortality observed in rearing the insects (Hodge, 1933) points toward this as well. Loss of secretory ability on the part of the cells on the oat diet, and especially in the villi, is evidenced by the lesser number of secretory inclusions, as well as by the approximation in dimensions of the cells of the villi to a size nearer that of the cells of the crypts.

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LICNOPHORA LYNGBYCOLA, A NEW SPECIES OF INFUSORIAN FROM WOODS HOLE

E. FAURÉ-FREMIET

(From the Marine Biological Laboratory, Woods Hole, Mass., and the Collège de France, Paris, France)

The genus *Licnophora* (Infusoria Heterotricha) includes some well characterized marine species; Kahl's revision of the group (1932 and 1933) is a convenient guide in their identification.

I have observed a species which does not seem to have been described yet. As a matter of fact, there is but little difference between it and the species already known in the morphological characters, but, in a biological sense, this new species is quite peculiar in its constant association with an alga from the group of the Cyanophycea, which it uses as a support.

I first found this species at Woods Hole in July, 1929, and found it again at Concarneau in July, 1936. In both cases, the cultures of this infusorian and of the alga on which it clings had grown on glass-plates, immersed in running sea water, either in the aquaria of the Marine Biological Laboratory at Woods Hole, U. S. A., or in the Laboratory's fish-ponds at Concarneau, France.

I shall describe this species under the name of *Licnophora lyngbycola*, n. sp.

Structure

The infusorian is 100 μ long, and equally wide. The general form is changeable, often globular. The peristome is very largely everted in the fully expanded infusorian. In this case the ventral side is limited by an anterior brim, nearly circular, slightly depressed, and limited itself by the adoral zone.

The protoplasmic mass constitutes a dorsal rounded lump, more or less prominent and more or less wrinkled. The body becomes narrower towards the lower part, taking the shape of a straight foot, only 20 to 25 μ wide, which then becomes wider once more, forming a cup-like organ of fixation.

The mouth is placed ventrally, in the inferior third part of the body, under the protection of a thin protoplasmic lip. The adoral zone, at first horizontal in the whole of its pharyngeal part, rises in a circular

curve along the inward left side of the peristome, runs along the upper edge, comes down again on the right side, and finally, by means of a more pronounced curve perpendicular to the axis of the body, it follows a sudden protoplasmic ventral prominence which stands off in front of the mouth, and stops short at the point where this prominence comes down towards the cytostome, stretching out the slender cytoplasmic lip, already mentioned.

The fixation apparatus consists of a kind of circular cup, almost 20 μ wide, sustained and edged by a skeleton ring, refracting and homo-

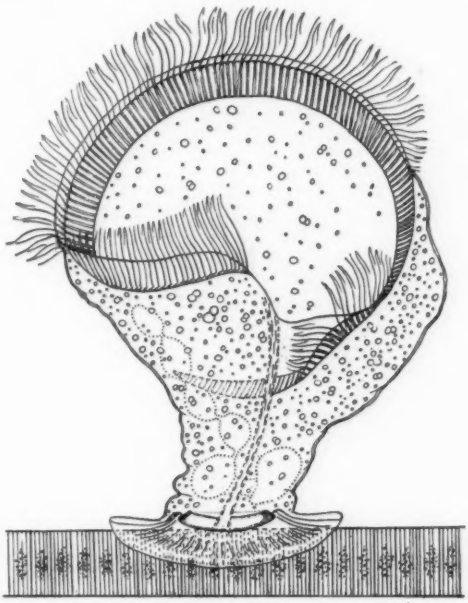

FIG. 1. *Lycnophora lyngbycola,* attached on the body of the Cyanophycea.

geneous; this ring, which is bent saddle-like, shows the most appropriate form for the fixation of the animal on the alga. Around it all there is a pedicular fringe, surmounted by a velum, that is to say, a slender and circular fold of the protoplasmic wall of the body.

A refracting, contractile fiber is fixed on the ventral side of the attaching disc and on the terminal edge of the adoral fringe, passing in front of the pharyngeal opening.

The cytoplasm is normally of the thready type, filled up with food

in the gastric vacuoles at different states of digestion, heaped up in the dorsal lump.

The macronucleus is beaded; it is made up of three rather large segments displayed in the manner of a horseshoe over the attaching disc, and of a series of approximately 8 or 10 smaller segments, which compose a semicircular row of beads, going upwards on the right side and stretching dorsally along the right and superior sides of the adoral zone. A spherical and single micronucleus is situated under the pedical segment of the macronucleus.

The protoplasmic body is enclosed by a rather rigid pellicle, with double outline, slightly and closely punctuated, more or less wrinkled,

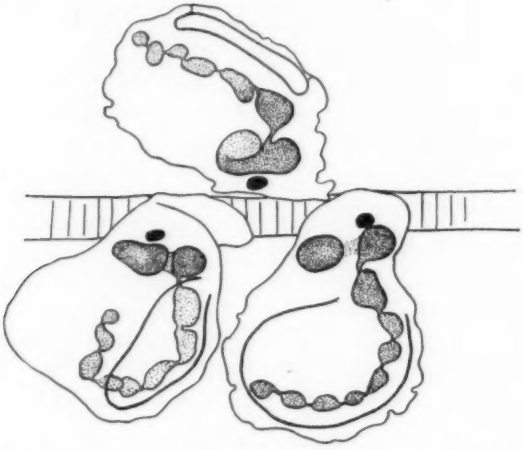

FIG. 2. Schema of three individuals, showing the beaded macronucleus (dotted) and the micronucleus (black).

and united by a protoplasmic net-work to the principal cytoplasmic mass. The infusorians of Woods Hole showed some polygonal plates in their pellicle, with joint-lines; but at Concarneau they were only stippled.

These infusorian individuals are found on the filaments of a colorless Cyanophycea which belongs probably to the group of the Lyngbyæ. These threads, fixed by one end on a solid support, stand up almost vertically, bearing the infusorians, sometimes one only, sometimes a group of ten or twelve individuals. As they change places nearly all the time, one can watch them as they move upwards and downwards, pushing one another. In making these movements, they bend their peristomes in all directions, and in normal conditions they will move all

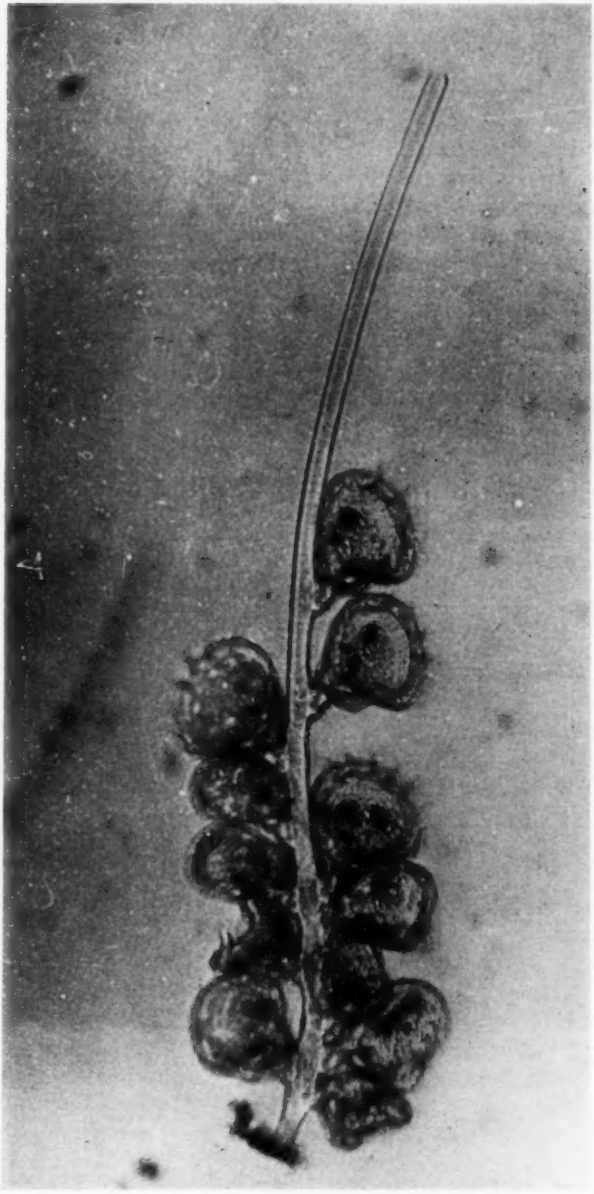

FIG. 3, *a* and *b*. Microphotographs of a group of *Lycnophora lyngbycola* on the Cyanophycea, in living state.

around the alga, without showing the slightest tendency to leave it. Now if, by a manipulation, you detach them mechanically, they begin to swim freely, but it seems that they are incapable of fixing themselves on any plane or even irregular surface, the attaching disc always maintaining its rigid and bent shape; this shows clearly the very close and typical adaptation of this infusorian to the Cyanophycea which bears it.

At first glance, a group of *Licnophora lyngbycola,* seen under low power, looks like a colony of *Epistylis.*

In another way, the general appearance of these infusorians, when in groups, fixed as they are on their rigid thread as if on a stalk, might be compared with a form found in the vegetable kingdom, the rose-mallow, *Althea* inflorescence.

Licnophora lyngbycola, n. sp., is a form rather close to *L. Kohni,* Clap. et Lach., considering the structure of the peristome, but it stands apart in regard to its very prominent dorsal lump, the bent cup of fixation, the shape of the macronucleus, and the very close adaptation to the vegetal support on which it lives.[1]

[1] For bibliography, refer to: Kahl, A., 1932. Wimpertiere oder Ciliata, in *Die Tierwelt Deutschlands.* G. Fischer, Jena; Kahl, A., 1933. Ciliata libera et ectocommensalia, in *Die Tierwelt der Nord- und Ostsee.* Leipzig.

SOME EFFECTS OF NUMBERS PRESENT ON THE RATE OF CLEAVAGE AND EARLY DEVELOPMENT IN ARBACIA

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Frank and Kurepina (1930) reported accelerated development for sea urchin eggs, species not given, when 10 to 20 eggs were present per drop as compared with the rate when only one or two eggs were present. They used hanging-drop cultures and determined the relative degree of development at varying times after fertilization. Apparently they did not examine early cleavages. They interpreted their findings as being the result of the action of mitogenetic rays. Allee and Dr. J. R. Fowler began the exploration of this problem at Woods Hole during the summer of 1931 and obtained preliminary results for *Arbacia punctulata* which indicated that fertilized eggs left standing in drops of varying size for 16 hours or longer showed a decided tendency toward faster development in the more crowded as compared with the less crowded populations. At times, however, isolated eggs would develop as rapidly and as perfectly as did those in the somewhat crowded cultures. In these preliminary and hurried experiments there was no steady difference in the time to 50 per cent cleavage for the first two cleavages which were the only ones studied.

The present authors took up this problem in the summer of 1934. The methods of handling the sea urchins, of procuring eggs and sperm and of fertilization were based closely on the directions given by Just (1928). Usually freshly collected animals were used one to two days after being delivered in our laboratory. Later in the season eggs and sperm were obtained from sea urchins which had been collected for some time and, by being kept in the laboratory, still retained their sexual products when those collected daily were mostly spent.

Specifically we dried the animals on paper towels, meantime pressing the spines out of the way and breaking several loose from the body. The urchins were then opened by cutting with scissors around the peristome; Aristotle's lantern was removed with tweezers and the edge of the peristome shell was broken somewhat. The body fluid was shaken out and the animals thus prepared were placed aboral side down in a

clean, dry Syracuse watch-glass. Normally eggs were soon shed by one or more of the females. These were removed after extrusion by pipetting through washed new cheese cloth into finger-bowls containing filtered sea water and were decanted through several changes of similar sea water. Shed sperm were selected according to Just's criteria, covered and left until needed. One drop of the "dry" sperm was diluted in 10 cc. of filtered sea water in a Syracuse watch-glass. At first this was used almost immediately but beginning with the hanging-drop experiments diluted sperm was allowed to stand for 2 to 3 minutes before being used to inseminate eggs. Fertilization was accomplished by adding two (or sometimes one, or three) drops of this suspension to the eggs in 250 cc. of sea water in a finger-bowl.

Early and late in the season when the *Arbacia* were not shedding readily, the ovaries were shaken over cheesecloth to obtain eggs. Late in the season, on a few occasions sperm were pipetted from ripe appearing testes which, however, were not shedding normally. Trial fertilizations came to be the rule; usually evidence from the fertilization membrane stage proved trustworthy. Relatively few experiments had to be discarded because of low percentage of cleavage. Eggs from one female only were used in any given comparison; usually eggs from one female sufficed for all experiments made in any given half-day. Late in the season eggs from a "good" female which had been opened about 9:30 A.M. were used for inseminations up to about 3:00 P.M. The fertilized eggs were allowed to stand 5 minutes and were then transferred to experimental conditions. All experiments were eliminated from consideration which did not show at least 95 per cent cleavage in the sparse populations and 90 per cent cleavage in the more densely populated drops. Usually the cleavage was well above 95 per cent in both.

When 90 or 110 per cent sea water was used in the experiment, the eggs were usually fertilized in this medium. At times, with these solutions, the eggs were fertilized in normal filtered sea water and centrifuged at the lowest speed shown by a power centrifuge for 15 seconds; this sufficed to throw them to the bottom. The supernatant sea water was poured off and the eggs were gently washed into the appropriate experimental medium.

After the first few days, initial handling of eggs and sperm was all done by Evans; two stop-watches were started by Allee at the moment of dropping the second drop of sperm suspension into the water containing eggs. All isolations and group transfers were made by Allee under a low power dissecting binocular. Typically, after standing five or six minutes, a part of the fertilized eggs were pipetted to a clean

Syracuse watch-glass recently rinsed with sea water. Using a hæmocytometer tube in which each of the ten units on the tube proper equaled 2 cu. mm., the approximate number of eggs needed were transferred to the experimental slides in drops of the selected size. Early in the work these transfers were made in the open air, sometimes, unfortunately, before open windows. Later all transfers were made to experimental slides temporarily housed in a moist chamber lined with wet filter paper. The separation was usually completed by 15 minutes after fertilization.

Cleaning

After the first few days, cleaning of glassware was done by Evans. The Syracuse watch-glasses into which the eggs and sperm were shed and the finger-bowls in which the eggs were washed and fertilized and all pipettes were washed in running cold tap water and boiled for at least 10 to 15 minutes. They were usually drained dry on paper towels. Occasionally when needed immediately they were dried with clean cotton towels. The hæmocytometer pipette used in isolations was similarly treated except that at times between isolations of different lots of eggs on the same half-day, it might be rinsed in fresh water and allowed to stand full of fresh water for as long as possible before the next transfer; frequently even under these conditions, this pipette was boiled. The eggs used in successive experiments were fertilized at least 15 minutes apart, hence any contamination could be easily recognized and discounted; very few occurred.

The glass slides were washed in warm soapy water to remove the vaseline and vacuum grease used in making seals. They were thoroughly rinsed in running tap water and were rinsed again in hot water and were usually boiled. They were polished with clean cotton towels.

Temperature

No attempt was made to control the temperature of the water in which the fertilizations took place. This water was usually the normal sea water freshly drawn from the laboratory tap, filtered and left standing in a large Florence flask. It was usually somewhat below air temperature at the time of fertilization. All observations were made in a room having north light only, hence there was no direct sunlight to interfere with room temperatures.

The small separate drops must have approximated air temperatures in a short time. These ran no higher than 25.5° at any time and were usually between 23–25°. When the air temperature was 20° or lower, the whole cleavage test was made at that temperature. In the hypertonic sea water experiments (110 per cent), room temperatures were

also used. At other times the experimental slides were placed in cooling chambers for as much time as possible between the setting up of the experiment and the second cleavage. These chilling chambers varied from a rudely insulated makeshift refrigerator to an elaborately regulated chamber. They were usually held at 17–19° and normally were fairly constant for any given experiment. In one experiment the temperature went as low as 16°. An attempt was made to arrange the temperature so that first cleavage would come after some fifty minutes and second cleavage after about eighty-five minutes. Isolations were completed in about fifteen minutes and the slides were placed immediately in the cooling chamber, if that was the indicated procedure. Except in early experiments, dense and sparse populations were on the same slide and were accordingly subjected to the same temperatures throughout an experiment; hence more exact temperature regulation was not needed.

Test for Accuracy of Determination of Fifty Per cent Cleavage

In our first experiments we undertook to determine cleavage on living eggs. Accordingly we determined the relation between the time recorded by one of us for 50 per cent cleavage in living eggs, using our usual technique, as compared with the time similar eggs cleaved as determined by spaced killings of eggs kept under the same conditions. The mean error of determination of 50 per cent cleavage in eight cases was 10 ± 3 seconds.

Observations

The following experimental conditions were used and in this order:

Paraffined Slides.—Microscope slides with a shallow hollow ground cell were coated with paraffin. Two grades were used. The first had a relatively low melting point. The second was the highest grade, highest melting-point paraffin available in Woods Hole. There was no marked difference in the results obtained with the two grades. Melted paraffin was painted on the slides just before they were to be used and was removed, preparatory to washing them, after each experiment. One drop of water containing eggs was placed in the center of each depression and covered by a thin, somewhat curved watch-glass vaselined around the edge to make a small moist chamber. Typically 12 to 14 such slides made an experimental set. One had a drop containing approximately 100 to 200 eggs. Another had approximately half that number. The remainder held about two each. One person followed the cleavage of the two denser populations under a low power of a compound microscope while the other, seated nearby, followed it for the

sparse populations. After the first experiment, the drops used contained 10 cu. mm.

These drops rounded into bead-like objects. Eggs near the periphery were sometimes difficult to see clearly. The slides which held the more densely populated drops were in the fingers of the observer more than was any one of the slides holding the sparser populations. This may have caused temperature differences between the two lots such as would favor the more rapid cleavage of the eggs in the former.

In the six experiments (see Table II) which meet the percentage of cleavage requirements, the time to 50 per cent first cleavage was 0.93 minutes and to second cleavage 2.17 minutes less in the denser populations of eggs than in the accompanying sparser populations. The statistical probabilities are 0.3 and 0.129 respectively. This means that neither of these differences are statistically significant.

Indications appeared that the paraffin retarded development. This was tested in two direct experiments in which approximately 21 hours after fertilization, the mortality was higher in the paraffined slides, particularly those with sparse populations. Accordingly this experimental method was discarded.

Clean Glass Slides.—The technique used resembled that just described except that no paraffin was used. With both of these sets of slides, it took about one minute to examine the lot of sparsely populated slides. Hence, to be significant, differences needed to be more than one minute provided all of the sparsely populated slides were to be inspected each time. Frequently as the critical time approached this was not the case since some of the slides would have all the few eggs present cleaved and could be discarded for the time being. In all, eight paired comparisons were made under these conditions. These showed a mean time to 50 per cent cleavage which averaged 1.53 minutes less time for the more crowded populations for the first cleavage and 2.2 minutes less for the second cleavage than for accompanying sparse populations (see Table II). These have statistical probabilities of 0.068 and 0.036 respectively. The latter is within the range of statistical significance as commonly interpreted.

Hanging Drops.—A central drop with from 11 to 1,000 eggs was surrounded by five similar drops holding in all from 6 to 28 eggs, usually about 10. As in the preceding series, the size of drops was kept uniform at 10 cu. mm. The drops were fenced from each other by vaseline lines to prevent their flowing together. Each slide, so arranged, was inverted over a salt cellar type watch-glass with ground glass upper surface which was vaselined to seal. A small amount of water in the bottom made an efficient moist chamber. This technique put sparse and

dense drops under the eye of the same observer and on the stage of the same microscope. About one minute was consumed in making one complete inspection as cleavage approached 50 per cent. As elsewhere, if the 50 per cent cleavage point was reached in both dense and sparse populations during the same cycle of observations both were recorded as reaching this stage at the same time. The eggs in these hanging drops were located in the surface film exposed to unknown stresses. This constitutes the chief objection to the method.

Fifteen experiments which met standard conditions gave a mean time to 50 per cent first cleavage of 0.57 minutes faster in the more sparsely populated drops and to 50 per cent second cleavage of 0.24 minutes for the same eggs. These differences have a statistical probability of 0.215 and 0.84 respectively and accordingly the results have no statistical significance (see Table II).

Assembled Moist Chambers.—The assembled moist chambers consisted of a glass base 4 cm. sq. etched in the center with lines 1 mm. or less apart to form squares which aided in counting the denser populations of eggs. The vertical wall was formed by a glass ring ground on both surfaces. This ring was about 3 cm. in diameter and 4 mm. high. It was sealed to the base with vacuum grease and a plain glass cover was sealed on with vaseline. The base usually had vaselined lines to keep drops from running together. In much of the work, the vaselined lines were arranged in circles which limited the surface of the drops to about the same area. In one type of experiment, the denser population was placed in the center; the five surrounding drops held normally two eggs each; in three cases there were as many as 15 to 23 eggs in one of these sparsely populated drops. The time consumed in making a complete cycle of observations was about that with the preceding methods. Later only two drops were used, one with a dense population which might run up to an estimated 8,000 in 20 cu. mm. Nearby, perhaps connected with the denser lot of eggs, perhaps isolated, would be a similar drop with a sparse population. The time from the determination of 50 per cent cleavage in the denser population until a count of cleavage could be made in the sparser one was usually 15 seconds or less. All the later experiments were made using this last method.

The seals on such assembled moist chambers had to be carefully made to avoid evaporation. These seals allow for ease of cleaning and the possibility of rapidly changing clouded covers. Evaporation from the experimental drops was frequently lessened by the addition of unoccupied drops of sea water in the free spaces. Even so, with the small drops and the change of covers made necessary by their clouding on

removal from cooling chambers (see below), some evaporation must have taken place. It is difficult to determine whether the evaporation is equally distributed through these small drops. The drop isolated first, other conditions being equal, should show the greatest amount of evaporation. There is evidence that hypertonicity is a potent factor in retarding the time of cleavage of echinoderm eggs and since in many of these experiments the eggs in the more densely populated drop tended

TABLE I

A summary of time to 50 per cent cleavage with two connected or closely associated drops, one containing a very dense and the other a sparse population. All were tested in normal sea water.

Crowded Drops				Sparsely Populated Drops						
No. Eggs	Minutes to Fifty Per Cent Cleavage		Percentage Cleaved	No. Eggs	No. Followed	Minutes to Fifty Per Cent Cleavage		Percentage Cleaved	Cu. Mm. in Drop	Temperature ° C.
	I	II				I	II			
3500	63.25	94.25	98+	17	17	64.83	97.50	100	10	18–25
4000	61.00	94.50	99+	130	28	62.25	96.67	99+	10	16–24
1600	67.83	100.04	99+	250	24	70.67	104.41	99+	10	16–24
2100	68.33	101.25	99+	11	11	70.75	103.41	100	10	16–24
2000	71.83	105.00	99+	41	21–30	74.00	108.08	100	10	16–24
1800	55.41	84.00	100	40	10	56.17	86.58	100	20	21–21.5
4000	52.17	80.50	100	800	10	60.25	90.25	100	20	21–21.5
5000	62.91	95.75	99	600	16	64.25	97.75	100	40	19
2500	58.67	—	99	800	16	61.00	—	100	40	19
6000	62.33	96.50	96	15	12	63.75	99.08	100	20	19–20
8000	61.25	95.00	96	17	12	63.25	97.25	100	20	19–20
6500	61.58	95.25	95	14	12	61.83	96.25	100	20	19–20
2800	61.33	94.58	95	23	12	61.17	94.83	100	20	19–20
3831	62.145	94.72	98	212	16	64.174	97.67	100−	19	

to cleave first, these were isolated first. Here and elsewhere whenever it was impossible to set up conditions exactly equivalent for dense and sparse populations, the sparser population was favored. This necessitated the isolation of the dense group last when hypotonic sea water was used, since under such conditions the longer the drops stood exposed to air, the more nearly they approached normal concentration.

Similar experiments were performed with normal sea water, with 110 per cent and with 90 per cent sea water. With the 110 per cent sea water no cooling was used to slow down development.

The results obtained with the two drop technique are given in some detail in Table I and all the work done with the assembled moist chamber

is summarized in the last four lines of Table II. In all these cases the time to 50 per cent second cleavage was significantly less for the more crowded eggs. The decreased time ranged from 2.17 to 3.16 minutes and the statistical significance from 0.0134 to 0.0002. As usual, there was less difference in the time elapsed before 50 per cent first cleavage. With 90 per cent sea water the results were slightly negative

TABLE II

A summary of differences in time to 50 per cent first (I) and second (II) cleavage with dense and sparse populations of Arbacia eggs.

No. of Experiments	Method	Mean Difference in Minutes		Statistical Probability	
		I	II	I	II
6	Paraffin	0.93	2.17	0.3*	0.129*
8	Glass slides	1.53	2.2	0.068*	0.036
15	Hanging drops	−0.57	−0.24	0.215*	0.84*
21–23	Moist chamber	0.54	2.7	0.194*	0.002
17–18	Do. 110 per cent	1.9	3.16	0.0084	0.0002
10	Do. 90 per cent	−0.03	2.17	0.67*	0.0134
12–13	Do. connected drops	2.03	2.95	0.0036	0.0014
True means and combined significance		0.88	2.23	0.053	0.0016

* Results taken alone are not statistically significant.

(0.03 minutes, probability 0.67). In two other sets of experiments this time difference increased to 1.9 and 2.03 minutes earlier cleavage for the more crowded drops with statistical probabilities of 0.0084 and 0.0036 respectively. The fourth comparison made (line 4, Table II) gave positive results which, however, lack statistical significance for first cleavage although there is good significance for the second cleavage.

The use of two connected drops, one with a dense and the other with a sparse population of eggs, sometimes permitted the observers to determine the time to 50 per cent cleavage in different regions with different densities. Two such determinations (in which Allee was observing and Evans was recording) are summarized in Fig. 1.

Averages of the time to 50 per cent first or second cleavage for the experiments summarized in Table II have lessened value because of the varieties of techniques used. The mean differences are based on individual paired experiments in any one of which the difference in density of the egg population was the only factor known to vary. If the true mean, considering the number of individual tests, is taken, there is a mean difference in time to 50 per cent first cleavage of 0.88 minutes

with the eggs in the denser populations cleaving first. This has a statistical probability of 0.053 which is slightly above the conventional limit of statistical significance. By second cleavage, this difference has increased to a mean of 2.23 minutes and the statistical probability has increased to 0.0016.

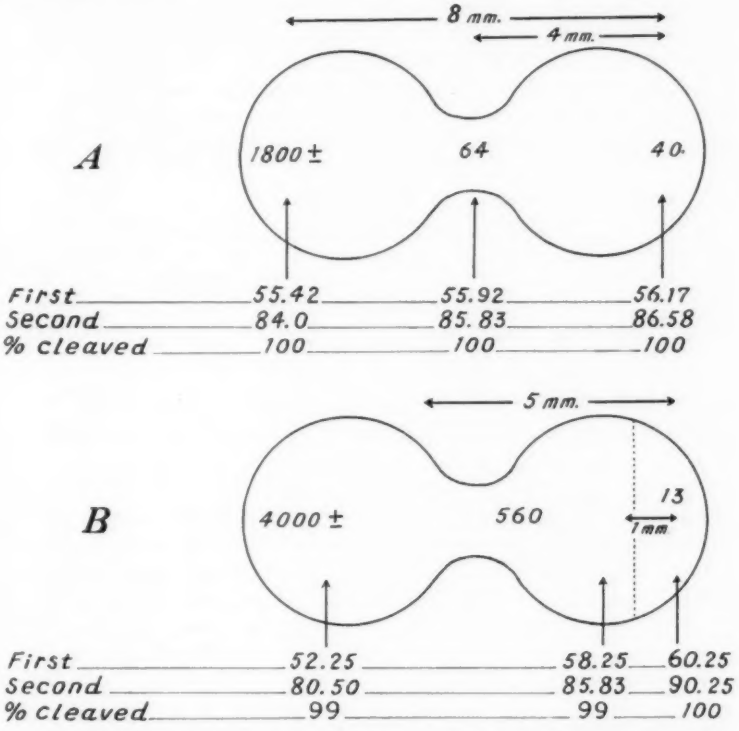

FIG. 1. Diagrams of the connected drops used in two different experiments. Each drop contained 20 cu. mm. The measurements give distances between points indicated by the double arrows. Figures below the diagrams, unless otherwise indicated, give time in minutes to the designated cleavage.

The values just given include those with hanging drops where the relations are obviously different from those obtaining in the other types of experimentation. If the hanging-drop experiments are omitted, these true means become 1.17 and 2.68 minutes with "p" values of 0.016 and 0.0000 respectively. Since these hanging-drop experiments do not support the evidence from the rest of the work to date and since

we do not know why this is the case, it is probably better to include these in the summaries. At least this is the conservative procedure and is in line with the general practise in these experiments of weighting the results against the experimental trend whenever it was impossible to be absolutely impartial.

Thus far in this account we have been dealing mainly with time relations with regard to 50 per cent cleavage. It is profitable to examine the assembled data from another approach which may be indicated by the following question: How many cases were there in which there was evidence of a shorter time elapsing between fertilization and 50 per cent cleavage in the denser population of eggs and in how many cases

TABLE III

Relations between first and second cleavages.

Evidence of Stimulation in Denser Population (D)	Without Hanging Drops	With Hanging Drops
1. D cleaved first in both cleavages	46	48
2. First cleavage a tie; D faster in second	24	27
3. D slower in I; faster in II	5	5
4. Sparse faster in I; second even	5	5
Totals	80	85
Evidence of Retardation in Denser Population		
5. S cleaved first in both cleavages	5	8
6. First cleavage a tie; S faster in II	0	5
7. D faster in I; S faster in II	0	0
8. S slower in I; second, even	1	2
	6	15
9. No observed difference	13	17
Totals	19	32

did the opposite hold true? The answers are given in Table III properly subdivided among the different possibilities. In the preparation of this table, unless there was a clear difference between the time to 50 per cent cleavage such that the difference could not be the result of error in observation, the case was recorded as showing no difference. Table III shows correctly that our data clearly indicate speedier cleavage in the more densely populated drops.

These experiments taken together demonstrate fairly conclusively that there is a difference in time to 50 per cent of first and second cleavage for *Arbacia* eggs in relatively sparse and in relatively dense population. The data collected are clearly statistically significant on this point

for the second cleavage, less so for the first. It is necessary to inquire into the degree of crowding which will bring about this result. Observations on this point were made from time to time during the progress of the experiments. Those obtained later in 1934, using the paired drop technique with one drop densely crowded with eggs closely associated or even actually directly connected by a narrow isthmus with a similar drop which contained but few eggs, are summarized in Table IV.

TABLE IV

Numbers present with relation to the crowding effect.

No. Experiments	Dense No. eggs	Sparse No. eggs	Difference in Fifty Per Cent Cleavage		Probability		Size Drops Cu. Mm.	Eggs Followed Sparse
			I	II	I	II		
12–13	1600+	800−	2.04	2.95	0.0036	0.0014	10–40	10–30
15	65–164	5–24	0.23	1.26	0.7	0.045	20	5–24
14	22–56	5–18	0.08	−0.3	0.38	0.426	20	5–18

In this table the difference in time to 50 per cent first and second cleavages are compared for crowded drops containing over 1,600 eggs as compared with that in more sparsely populated drops containing 800 eggs or less. In reality the listing of these hundreds of eggs in the sparser populations is hardly fair for in all cases the eggs watched in such drops were out of contact with each other while those in the densely populated drops were closely crowded. Under these conditions (which have already been discussed in some detail) the eggs in the more crowded drops cleaved significantly earlier than did those in the accompanying sparsely populated drops of equal size. When the more crowded drop contained but 65–164 in a 20 cu. mm. drop and the accompanying drop held 5–24, the difference in time to first cleavage was insignificant but the denser eggs cleaved slightly sooner at second cleavage and the difference of 1.26 minutes is just within the upper limit for statistical significance. When the population in the more dense drop is still further reduced and that in the sparsely populated drop remains about the same, the difference disappears for both cleavages.

Factors Known to Retard Cleavage

The factors known to retard cleavage which may have operated in one or more of these experiments are:

1. Hypertonic sea water.
2. Lowered temperature.

3. Contamination with cœlomic fluid.
4. Contamination with fragmented eggs.
5. Increased amount of metabolic wastes in the water.

Of these, there was no opportunity for differential contamination with cœlomic fluid in the different parts of a given test. The precautions taken against hypertonicity have already been outlined and while they were not always successful when differential treatment was unavoidable as in the time of setting up of the various drops, especial care was taken to load the experiment against the trend of experimental findings.

As regards temperature differences, when one observer examined the one or two slides holding dense populations and the other handled the half dozen or more with sparser ones, there was the possibility of the former having a slightly higher temperature from the more steady contact with the fingers of the observer. This possibility was eliminated in the tests with the assembled moist chambers which constitute the majority of the experiments reported here. Furthermore, these latter experiments yielded greater and more consistent differences than were found under conditions that might have been suspect, hence there is no evidence that the observed differences are the result of an externally induced temperature differential.

There is, however, a possibility that the high rate of oxidation of the larger number of newly fertilized eggs confined in a small space may produce a temperature differential sufficient to account for the observed results. No tests of this possibility have been made to date. The best evidence that can be cited in its favor is that a slight differential increase in temperature would produce the difference which we have found.

The isolated eggs were definitely favored as regards the presence of fragmented or immature eggs. Allee found it psychologically difficult to select other than good eggs for the relative isolation of the sparse lots while in scooping up from 50 to 8,000 eggs for a more densely populated drop, no such selection was possible. Hence the sparsely populated drops contained less debris both absolutely and proportionally than did those with the large number of eggs.

Overcrowding.—In the most densely populated drops used in the regular experiments, eggs were present in about the proportion of 0.5 cc. of centrifuged eggs (lowest speed with power centrifuge for one minute) to six cc. of water. In Syracuse watch-glasses with this concentration there was reduced cleavage. In drops of 10 or 20 cu. mm., the eggs are nearer to the surface of the drops and while they may be piled three or four deep in the center, cleavage goes almost as well as in the

finger-bowl controls. When the same concentration is placed in a watch-glass or finger-bowl, i.e. in the same concentration when the eggs are all stirred up, the eggs settle to the bottom in a much more dense mass than any we worked with on the slides and under these conditions they were definitely over-crowded.

Mass Protection.—In an attempt to avoid the added evaporation incident to changing clouded covers, a microscope stage-cooling device was developed. This consisted of a brass quadrangle about the length of the microscope stage and somewhat narrower and about three centimeters high. Glass tops and bottoms were sealed on with DeKotinsky cement. The upper glass plate had patches of etched lines to facilitate counting and accommodated two of the 3 cm. glass rings which were sealed to the glass with vaseline. Intake and drain tubes were soldered to the brass quadrangle and were attached to the sea water supply since this was the coolest water available. This was further cooled by running it through a copper coil placed in a bucket of ice water. The drops were placed on the glass as usual except that during isolation, a covering watch-glass was used for a partial moist chamber. After covering the drops with the usual vaseline-sealed, glass cover, the water was turned through the chamber and the temperature fell. With the room temperature at 24°, in one instance, water emerging from the cooling device showed 12°; it was usually held at from 17–19°. This device was discarded when it appeared that there was difficulty in proper cleaning of the surface to which the eggs were exposed and no data secured by this means have been included in the preceding tables.

Some twelve experiments were tried using this gadget. The mean difference to 50 per cent cleavage for those that cleaved was 5.0 minutes for first and 5.62 minutes for second cleavage, with "p" values of 0.024 and 0.0052 respectively. Four accompanying experiments made in the assembled moist chambers already described had differences to 50 per cent first and second cleavages of 0.31 and 2.8 minutes respectively with the latter difference statistically significant. In two cases with the stage-cooling device, sparse populations of 6 and of 10 eggs failed to cleave and in two other experiments sparse lots of 24 and of 34 did not reach 15 per cent cleavage. The accompanying densely populated drops developed to or beyond the blastula stage and in three of the four comparisons just made, many in the more crowded lots were actively swimming after 24 hours. These data strongly suggest the presence of some toxic contaminating substance which was not completely removed by the methods used in washing these stage-cooling devices. Further tests showed that in connected drops gradients of resistance could be demonstrated which depended on the numbers present, the more eggs within

the limits tested, the greater the percentage of cleavage and the further development would proceed before death. Such mass relations appear to be closely related to the mass protection from toxic materials which has been repeatedly demonstrated (*cf.* Allee, 1931, 1934).

Could this have been the explanation of the more rapid cleavage observed in the denser populations? There is a suggestion that it may have been a factor in the paraffined slides, which, however, is not borne out by comparative studies unless there was some other toxic agent acting similarly in the remainder of the experiments. To suppose that there was mass protection from toxic materials in the other experiments would imply either some sort of toxic emanations from the glass itself or from some chemical previously in contact with it (Richards, 1936), or that traces of the vaseline and/or vacuum grease remained over from the washing and were poisonous, or that some of the soap from the mild suds used remained after the extensive and careful rinsing. There is no evidence for the presence of toxic materials from any of these sources all of which were considered as possible means of experimental error before this set of experiments was begun. Direct tests made by using water which had stood over masses of broken glass showed no difference in development as compared with similar cultures in ordinary sea water. Direct tests for toxic effects from vaseline and from the vacuum grease used made by coating slides with these substances and placing drops with different numbers of eggs on them, yielded no evidence of contamination from this source. Hence we concluded (and later experiments to be reported in another paper justify the conclusion) that the differential results obtained are not produced by mass protection such as was demonstrated to be operating in the experiments made with the stage-cooling device.

There is also internal evidence from the experimental results reported in Table II that something more is happening in these experiments than would be expected from the simplest of mass relations whether mass protection or otherwise. The data given there show that the mean time to 50 per cent first cleavage for all the experiments was 0.88 minutes (omitting hanging-drop experiments, 1.17 minutes) and to second cleavage was 2.23 minutes (omitting hanging drops, 2.68 minutes). The mean time to 50 per cent first cleavage was 57 minutes and to the same stage in second cleavage was 85 minutes.

If this were a case of simple mass relationships one would expect the acceleration of 0.88 minutes during a period of 57 minutes to continue at the same rate during the following 28 minutes to second cleavage. At this rate the total acceleration would approximate 1.31 minutes,

which is not the case. Even when one corrects for the fact that for the first ten minutes after fertilization, on the average, the eggs which are to make up the isolated or sparse populations are in fairly dense lots, the expected acceleration on the basis of uniform, simple mass action would be 1.40 which even yet is far from the observed value. Even omitting the hanging-drop experiments, which we decided above not to do, and using the basis of calculation which will give highest results, simple mass action would call for an acceleration by second cleavage of 1.87 against the observed value of 2.68 minutes. These relationships indicate that the observed phenomena are not based directly on the simplest sort of mass physiology.

Other possible causal factors which deserve investigation include the effect, if any, of increased carbon dioxide, of supernatant water from eggs both before and after cleavage, and of mitogenetic rays.

Summary

1. Other conditions being equal and under a variety of experimental conditions, eggs of *Arbacia punctulata* cleave more rapidly when in relatively dense as opposed to relatively sparse populations. The decreased time to first cleavage in the dense populations was 0.88 minutes and to second cleavage was 2.23 minutes. The first difference is probably not statistically significant; the second value is clearly significant.

2. Among other conditions, these relations were observed when some thousands of eggs in a drop of 20 cu. mm. of sea water were connected by a narrow strait with a similar drop holding some few tens or even a few hundreds of eggs.

3. If the eggs were crowded together too densely, the time to first and second divisions was definitely retarded and the percentage of final cleavage was reduced.

4. When 22 to 56 eggs were placed in one drop of 20 cu. mm. connected by a strait with another containing 5 to 18 eggs, no difference in cleavage rate was observed.

5. The observed differences are not a result of differential temperatures externally imposed, differential hypertonicity or hypotonicity, contaminations with cœlomic fluid or with fragmented eggs.

6. Such results may be obtained by mass protection from toxic materials. There is, however, no indication that differences here reported were so caused.

7. Although no supporting evidence is presented here, the results may conceivably have been the result of differential temperatures produced by the high rate of oxidation of the massed eggs in a small space,

by chemical emanations from the eggs, including carbon dioxide, by stimulation from mutual contact or by mitogenetic rays. Discussion of these problems is reserved for the present.

8. In addition to their intrinsic interest, the results provide another instance of physiological activities which proceed more rapidly at an intermediate optimum than when either too few or too many are present.

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FEEDING RATE OF CALANUS FINMARCHICUS IN RELATION TO ENVIRONMENTAL CONDITIONS[1]

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(From the Woods Hole Oceanographic Institution and the Biological Laboratories, Clark University)

OBJECTIVES

The amount of food available to a plankton-feeding animal is determined by the concentration of suitable food material in the water, and by the rate of the animal's feeding activity. The calanoid copepods, like many other zoöplanktonts, are generally considered to feed by filtering out particles from a current of water generated by the animals (Cannon, 1928). By determining the number of food organisms removed by an animal from a suspension of known concentration, the volume of water which has been filtered clear of the organisms can be calculated and, if the chemical composition of the organisms be known, the amount of nutriment made available to the animal may be estimated. Preliminary measurements of the filtering rate of *Calanus finmarchicus* have been reported by Fuller and Clarke (1936). Further investigation of changes in the feeding rate induced by varying environmental factors of ecological importance was expected to yield information useful in the quantitative study of aquatic food cycles. Diatom concentration, light and temperature were expected to be variable factors in the sea and were chosen for study.

METHODS

In the experiments here reported the rate of feeding of the copepod *Calanus finmarchicus*, collected in Vineyard Sound near the whistle buoy, was measured in a suspension of the diatom *Nitzschia closterium* (Plymouth strain). It seems probable that *Nitzschia* is a suitable food for *Calanus*, as Crawshay (1913–15) kept individual copepods alive for as long as 80 days in persistent cultures of this diatom. Allen and Nelson (1910, p. 470) reared *Calanus* from eggs to copepodid stages in a mixed culture in which *Nitzschia* was predominant.

Three stage V *Calanus* were placed in 15 cc. of sea water containing a known concentration of diatoms. Changes in concentration were followed for two to four days, counts being made in a hemacytometer.

[1] Contribution No. 132 of the Woods Hole Oceanographic Institution.

A series of counts on successive samples showed fair agreement with the theoretical Poisson distribution. The small excess variation found is attributed to the difficulty of shaking the diatoms to obtain even distribution without injuring the copepods. Enough diatoms were counted for each sample to give a result statistically valid to between 5 and 10 per cent. Under the experimental conditions this is the maximum precision obtainable. In these experiments a counting error of 10 per cent causes a discrepancy of about 30 per cent in the estimation of the filtering rate. Obviously only large variations can be satisfactorily studied.

TABLE I

Feeding rate at different diatom concentrations

Expt. No.	Date begun	Cells per cc.		Duration	W_{24}	ΔN_{24}
		C_1	C_2			
				hours	*cc.*	
47	Aug. 18	1,875	1,140	42	1.32	2,100
48	Aug. 18	1,875	1,155	42	1.28	2,060
63	Aug. 23	19,000	15,400	38	.66	11,400
64	Aug. 23	19,000	13,100	38	1.17	16,100
14	July 9	64,700	27,000	81	1.30	55,000
15	July 9	64,700	27,700	81	1.25	55,000
71	Aug. 26	190,000	147,000	38	.81	136,000
72	Aug. 26	200,000	133,000	38	1.29	212,000
11	July 3	335,000	86,000	84	1.61	356,000
10	July 3	375,000	220,000	84	.43	221,000
8	June 29	390,000	130,000	84	1.32	371,000
7	June 29	410,000	230,000	84	.60	256,000
				Average	1.09	

Calculation of the amount of water, W_x, swept free of diatoms by each copepod in x hours was made by means of an equation derived as follows:

$$dN = -dWC \tag{1}$$

$$dC = dN/V \tag{2}$$

$$dW = VdC/C \tag{3}$$

$$W_x = V \ln C_1/C_2. \tag{4}$$

N represents the number of diatoms per copepod; V the volume of water per copepod; C_1 and C_2 respectively the concentrations of diatoms at the beginning and end of the period of x hours.

The number of diatoms eaten in x hours per copepod, ΔN_x, is given

by the equation:

$$\Delta N_x = V(C_1 - C_2). \tag{5}$$

Parallel control suspensions were always counted to determine whether the diatom numbers were varying independently. If a control changed greatly during the experimental period, the results of the corresponding experimental series were discarded. If the change was small, the final concentration of diatoms in the control was substituted for C_1 in equations (4) and (5). This substitution assumes that the diatoms in the experimental containers increased or decreased independently exactly as those in the control. This is, of course, only an approximation.

Observations were also made on the production of fecal pellets. Since these pellets vary greatly in size no quantitative relationship could be obtained between the number of diatoms ingested and the number of pellets ejected. However, the formation of these excreta was a useful check on the reduction of diatom numbers observed directly.

Effect of Changing Diatom Concentration

Table I gives the results of experiments with different concentrations of diatoms. In the lowest concentration the diatoms were concentrated by the Nielsen-von Brand (1934) method before counting. These experiments were carried out at 13° C., and the animals were shielded from direct sunlight. The values for W_{24} and N_{24} in the table represent means over a period of several days. Diatom concentration often remains stationary for a day or more, thus indicating that feeding is not a continuous process. The maximum rate of filtration is higher than these values. The highest rate observed was in Experiment 11 where over a period of 14 hours W equalled 2.87 cc. which would give a W_{24} value of 4.9 cc. There appears to be no correlation between the concentration of food and the filtering rate. A comparison of the filtering rates of animals collected at different times during the summer yields no greater differences than a comparison of animals collected at the same time and used in parallel experiments (e.g. Nos. 10 and 11). The indication is that *Calanus*, though it sometimes does not feed actively, under otherwise constant conditions filters a definite volume of water per day, and obtains nutriment in direct proportion to the concentration of food particles.

Effect of Light on Feeding Rate

In certain experiments there appeared to be a diurnal feeding rhythm, removal of diatoms taking place most rapidly at night. This

is in accord with the observation of Marshall (1924), who notes that *Calanus* captured by tow-net hauls in the early morning had full guts, while those caught later in the day often had no food in their alimentary tracts. Figure 1 represents the course of two experiments (8 and 11) which show this rhythm. It is not, however, an invariable

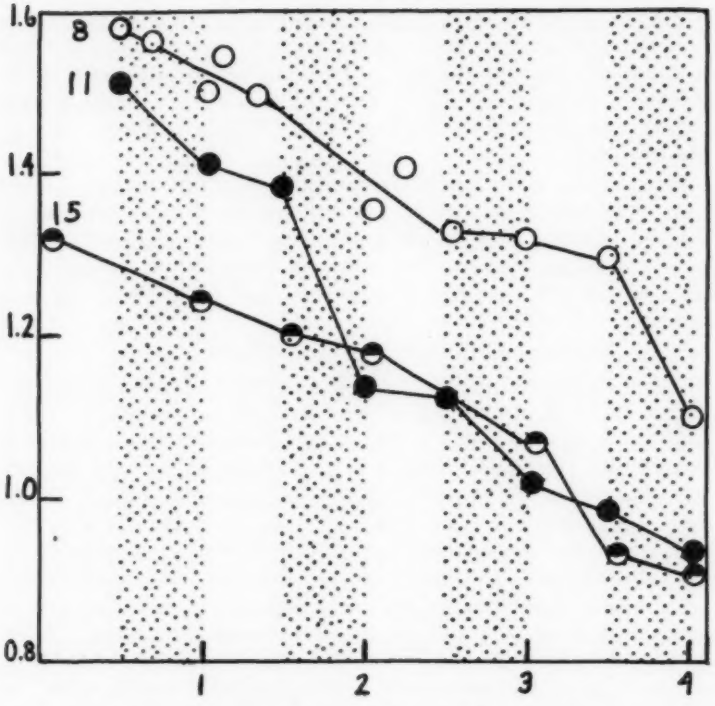

FIG. 1. Diurnal feeding rhythm of *Calanus*. Stippled areas represent hours between 8 P.M. and 8 A.M. The scale of Experiment 15 is adjusted by adding 0.5 to logarithms of cell numbers. Ordinates: *Logarithm-cells per 0.1 cu. mm.* Abcissæ: *Time in days.*

effect, as is shown by the course of Experiment 15, also shown in Fig. 1. Marshall likewise found that during the summer feeding occurred during the day as well as at night.

Experiments 26 and 32 differed from those reported above in being kept in darkness. Table II summarizes the results of these two experiments.

TABLE II

Feeding rate in dark

Expt. No.	Date begun	Cells per cc.		Duration	W_{24}	N_{24}
		C_1	C_2			
				hours	*cc.*	
26	July 16	54,000	32,000	90	.27	45,000
				48*	.76*	
32	July 30	61,000	20,000	65	1.8	76,000

* Animals actually fed only during last 48 hours of experiment. W_{24} value calculated for this period.

The results do not differ significantly from the average given in Table I, although there are admittedly too few cases to permit a definite answer. An attempt was made to study the effect of continuous light. The animals did not feed during the two days they were observed, but they were old stock in poor condition, and no reliance should be placed on this observation. Unfortunately it was impossible to collect more *Calanus* to make satisfactory experiments.

EFFECT OF TEMPERATURE ON FEEDING RATE

Experiments were carried out at 8° C. and at approximately 3° C. The latter temperature was not precisely controlled as the containers were placed in a refrigerator in general laboratory use. Both series were kept in the dark.

Feeding is much slower at 3° but still goes on with the formation of small compact fecal pellets. The very low filtering rates observed in Experiments 75 to 79 may be explained by the fact that the animals used were collected late in the season when the *Calanus* population was receding rapidly, and had perhaps stopped eating. The filtering rate in Experiment 80, in which the same stock was used, is seen to be much slower than that in Experiments 55 and 56. The high value for W_{24} in the latter experiments seems to indicate that 8° C. is somewhat more favorable for feeding than 13°. Excluding Experiments 75 to 80, the average values of W_{24} at 3°, 8° and 13° are respectively, 0.35, 2.83 and 1.09 cc.

ORGANIC NITROGEN CONTENT OF NITZSCHIA AND OF PLANKTON

Through the kindness of Dr. Theodor von Brand, an analysis of the organic nitrogen content of *Nitzschia* was made by the method he has developed for small amounts of plankton (von Brand, 1935). *Nitzschia* contains 0.9 microgram of nitrogen per million cells. Assuming the carbon-nitrogen ratio in this diatom to be equal to that

generally found in marine phytoplankton, approximately 8 : 1, we have 7.2 micrograms of carbon per million cells. Analysis of the fecal pellets of *Calanus* fed on *Nitzschia* showed that 0.55 microgram of nitrogen appeared in the feces for each million cells ingested. Roughly, about half the organic nitrogen was retained by the copepods.

Marshall, Nicholls and Orr (1935) have calculated from measurements of oxygen consumption the daily nutritive requirement of a stage V *Calanus* in summer as equal to 13 micrograms of carbohydrate. Assuming all the nitrogen of *Nitzschia* to be in protein, and the excess carbon to be in fat, one may calculate the number of cells which would supply an equal amount of energy. If the protein is assumed to contain 16 per cent nitrogen and 50 per cent carbon; the fat to contain 75 per cent carbon, we have:

TABLE III

Effect of temperature on feeding rate

Expt. No.	Temp.	Date begun	Cells per cc.		Duration	W_{24}	N_{24}
			C_1	C_2			
	°C.				hours	cc.	
42	3°	Aug. 18	263,000	215,000	90	.38	64,000
43	3°	Aug. 18	263,000	223,000	90	.32	53,000
55	8°	Aug. 22	85,500	30,000	42	2.88	159,000
56	8°	Aug. 22	85,500	31,000	42	2.78	156,000
80	8°	Aug. 31	24,000	14,000	65	.98	18,500
(75, 76) (79)	8°	Aug. 31			85	.00	0

$0.9 \times 0.16 = 5.62\ \gamma$ protein containing $2.8\ \gamma\ C$.
$7.2 - 2.8 = 4.4\ \gamma\ C$ in fat
$4.4 \times 0.75 = 5.87\ \gamma$ fat.

Proteins and carbohydrates have equal energy values per unit of weight. Fat has 2.25 as great an energy content as carbohydrate. Calculating fat and protein in terms of carbohydrate, we obtain:

$5.87 \times 2.25 = 13.2$
5.6
$18.8\ \gamma$ · weight of carbohydrate equivalent in energy content to a million *Nitzschia* cells.

$\frac{13}{18.8} \times 1{,}000{,}000 = 690{,}000$ number of *Nitzschia* cells which would contain theoretical food requirement.

The maximum amount of water filtered per day (W_{24}) in any experiment was 2.88 cc. A concentration of 240,000 cells per cc. would be required if *Calanus* is to obtain its theoretical food requirement from this volume of water, even if all the organic matter is utilized. Probably this value should be doubled since only half the nitrogenous organic matter appears to be assimilated. In any case this is a much higher concentration of phytoplankton than ever occurs in nature, but since the size of phytoplankton cells varies so greatly, comparisons based on cell numbers are worthless. It is, however, possible to determine the organic nitrogen content of the particulate matter in sea water, and to compare this with the food requirements of *Calanus*. Table IV summarizes the results of two sets of analyses by Dr. von Brand of the particulate matter suspended in Vineyard Sound water. All macroscopically visible organisms were removed

TABLE IV

Organic N in particulate matter in Vineyard Sound water

Date	Depth	Temperature	N
	meters	*° C.*	*per liter micrograms*
July 2	0	17.8	15
	15	13.5	27
	30	9.1	13
August 14	0	20.0	38
	15	18.0	42
	30	12.0	19

from the August 14 samples before analysis. This was not done for the July 2 samples.

The maximum value found was 42γN per liter, a nitrogen concentration equal to that of a *Nitzschia* culture containing 46,700 cells per cc. This is so far above the average number of diatoms per cc. in Vineyard Sound—the figure is probably close to 100—that, even allowing for the fact that one large cell is the equivalent of many *Nitzschia*, it appears probable that the major portion of the particulate matter at this station was not in the form of diatoms. The remainder, detritus or other organisms, is presumably of great significance as a source of food.

Even in this region of high nitrogen content 2.88 cc. of Vineyard Sound water contained less than one-fifth the theoretical food requirement, and in all probability not over one-tenth this amount since half the nitrogenous matter is believed inassimilable, and some of the particles were probably too large or too small to be captured. Yet *Calanus* survives and grows in this region. Two explanations are

possible: (1) *Calanus* in nature requires less food than that calculated from laboratory experiments on oxygen consumption, or (2), *Calanus* in nature filters a greater volume of water and thus obtains more than the estimated amount of food.

Nutritive Requirements of Calanus

Knowing the rate at which *Calanus* feeds on *Nitzschia*, it should be possible to supply the animals with known concentrations of

Table V

Survival and moults of Calanus fed on Nitzschia

Expt. No.	Nitzschia cells/cc.	Initial population		Successful moults	Deaths in moult	Deaths not in moult	Percentage reaching moult	Average survival
		No.	Stage					
								days
B1	300,000	2	III	0	2	0	63.2	8.9
		12	IV	1	8	3		
		5	V	0	1	5		
B2	150,000	11	IV	1	3	7	33.3	9.7*
		4	V	0	1	3		
B3	30,000	14	IV	0	5	9	38.9	9.5
		4	V	0	2	2		
B4	15,000	8	IV	0	3	5	41.1	10.4*
		11	V	0	5	5		
B5	0†	10	IV	0	1	9	18.7	11.0*
		6	V	0	2	3		
B6	Harbor water	4	IV	0	3	1	60.0	7.2
		16	V	0	9	7		

* In each of these experiments one stage V still living after 19 days.

† All attempts to moult near end of experiment. Examination showed minute protozoa present. None in Experiments B1–B4.

diatoms, observe the minimum concentration at which growth takes place, and calculate the food requirement in terms of planktonic organic nitrogen. Experiments were set up in which 15 to 20 *Calanus* were placed in 250 cc. of Berkefeld filtered sea water containing definite concentrations of *Nitzschia*. The concentrations chosen together with the amount of nitrogen per cc. in each case were:

(B1) 300,000 cells and 0.27γN per cc. representing a concentrated food supply with the theoretical amount of nutriment contained in 2.5 cc.

(B2) 150,000 cells and 0.135γN per cc., half the above.
(B3) 30,000 cells and 0.027γN per cc. representing an approximation to the concentration of organic nitrogen found in Vineyard Sound.
(B4) 15,000 cells and 0.014γN per cc., half the above.
(B5) Berkefeld filtered water—no diatoms.
(B6) Water collected from Woods Hole Harbor.

The medium was changed daily except Sunday, and dead animals were removed at each observation period. Animals were transferred

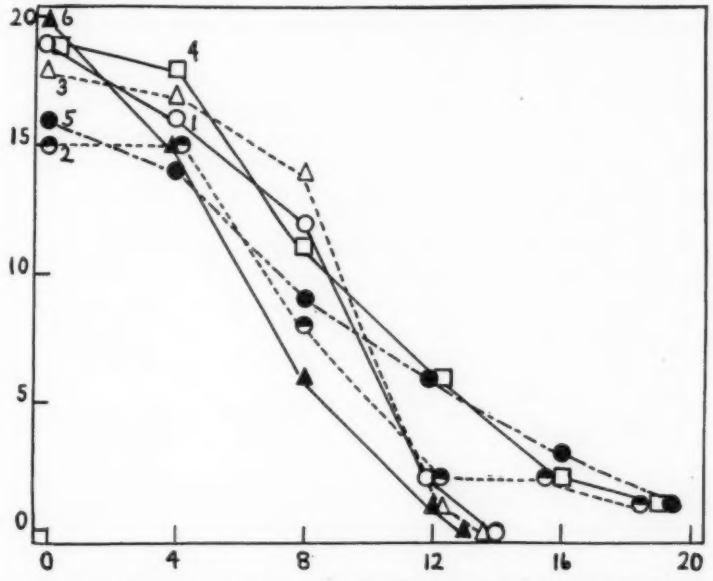

FIG. 2. Series B. Survival of *Calanus* fed on different concentrations of *Nitzschia*. B1—300,000 cells/cc.; B2—150,000 cells/cc.; B3—30,000 cells/cc.; B4—15,000 cells/cc.; B5—Berkefeld filtered water; B6—water from Woods Hole Harbor. Ordinates: *Number of survivors*. Abcissæ: *Time in days*.

by means of a lifter of bolting cloth or in a wide-mouthed pipette. In order to study growth, stage IV *Calanus* were used insofar as they were available. Survival was uniformly poor compared with experiments which were reported previously by Fuller and Clarke.[2]

These experiments are summarized in Table V and Figs. 2 and 3.

[2] Clarke and Zinn found that in both 1935 and 1936 there was a sharp reduction in the *Calanus* population in Vineyard Sound during the latter part of August. The animals were either being carried away, or were dying off. This may explain the high mortality in these experiments.

The previous observation that ecdysis is a critical period for *Calanus* is again brought out by the data for "deaths in moult." It is believed, however, that the attainment of the moulting stage represents true growth, and indicates that the animals are feeding, since ecdysis does not occur in starved animals (Fuller and Clarke, p. 312). If the attainment of the moulting stage—whether successful or not—be taken as the criterion, growth is most rapid in the most concentrated diatom suspension and in harbor water in which media 63.2 and 60 per cent, respectively, of the original population reached the moulting stage. Growth rates in the other diatom suspensions are essentially equal

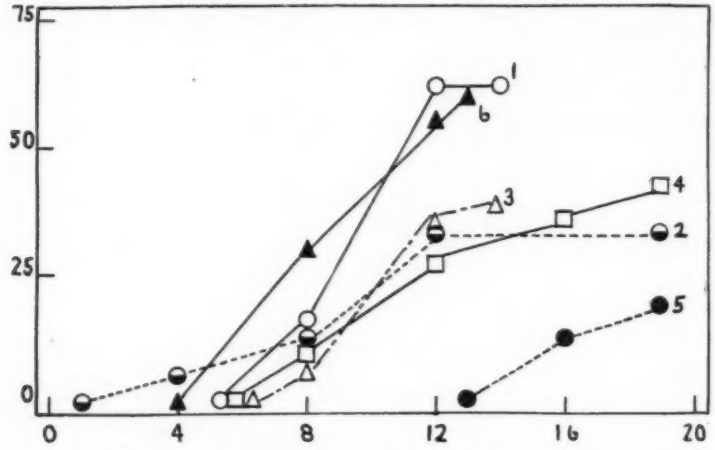

FIG. 3. Series B. Percentage of original population of *Calanus* attaining moulting stage when fed different concentrations of *Nitzschia*. Symbols as in Fig. 2. Ordinates: *Percentage of moults*. Abscissæ: *Time in days*.

(33–41 per cent), while the few moults which occurred in Berkefeld filtered water (19 per cent) are probably due to a protozoan infection of the culture which developed during the last few days of the experimental period and furnished a food supply for the copepods. There is a definite negative correlation between the average survival period in each experiment and the percentage of animals attaining the moulting stage. This is attributed to the fact that when growth is slower, the critical period of moulting is reached later and average survival increases.

If each *Calanus* captured daily the diatoms from 2.5 cc. of water, then in Experiment B4 in which some growth obviously took place, each individual obtained per day only 37,500 cells, 0.034γN, and

between 8 and 16 per cent of the food requirement calculated from oxygen consumption. The higher value assumes all the nitrogenous matter to be utilized; the lower value is based on the assumption that one-half is utilized.

Discussion

The filtering rate of *Calanus* in these experiments was less than that found by Fuller and Clarke using carmine particles, the previous value at 13° C. being 5.61 cc. per day against 1.09 cc. reported above. As the carmine particles tended to settle and were more difficult to count, the present values are the more reliable. Harvey (private communication) has found filtering rates of from 50 to 100 cc. per day using the larger diatoms *Lauderia* and *Ditylium*, though his values are based on only a few experiments. It seems probable that large particles are captured more readily, though the carmine particles mentioned above were somewhat smaller than *Nitzschia*.

Whether these varying results represent actual differences in the rate of filtering is a moot point. Possibly the variance depends upon the efficiency with which the maxillary bristles strain out particles of different sizes and shapes. It may be that small cells, such as *Nitzschia*, pass between the bristles so that a copepod retains only a small percentage of the *Nitzschia* in the water current which it generates. This is the author's view. On the other hand some investigators believe that copepods possess other food-capturing mechanisms besides filtration.

Lowndes (1935), basing his opinion on anatomical grounds, and on observation of living unrestrained animals, states that feeding of the calanoid copepods cannot be merely automatic, non-selective filtering, but involves the finding and grasping of food particles. *Calanus* has two distinct types of movement: (1) saltation in which propulsion is furnished by the antennules and swimming feet, and (2) a slow glide in which the motive force comes from the vibration of the mouth parts. It is the latter which furnishes the feeding current. Often this species remains suspended quietly in the water for several minutes. Since the eye of copepods is not capable of forming images, recognizance of food material would have to be chemical or tactile in nature. In a turbulent fluid medium, only tactile stimuli would be serviceable in precisely locating food. Saltations might enable a copepod to try out different regions for food, the filtering mechanism being called into play only when food was encountered. One would thus expect more filtering where food was abundant since more particles would be encountered. On the other hand, the rate of feeding might be controlled by the amount of food ingested, so that filtering would slow

down when food was abundant and speed up when food was scarce. No tendency towards a change in the filtering rate was shown. Harvey's results agree in this respect. Two conclusions are possible: (1) the rate of filtering is independent of the amount of food ingested or encountered; (2) even in the highest diatom concentrations used in these experiments there was insufficient food so that filtration was maintained at a maximal rate. Since in many experiments (e.g. 7 and 8) diatoms were present in concentrations much higher than are ever found in nature, alternative (2) is ruled out. The evidence definitely favors the theory that filtration is an automatic process independent of the concentration of food, though its rate is modified by physical factors in the environment.

These factors insofar as they have been studied may be considered in relation to the ecology of the species. The suggestion of a diurnal feeding rhythm is nicely correlated with the habit of vertical migration in this species which rises to the surface at night, and sinks below this rich food zone during the day. Neither phenomenon is absolutely regular. Probably both are dependent upon the physiological state of the animal which conditions its response to light (see Clarke, 1934). The ability of *Calanus* to feed at temperatures as low as 3° C. and its high rate of filtering at 8° indicate a physiological adaptation to life in the colder parts of the ocean.

The question of the food requirement of *Calanus* is perhaps of greatest fundamental importance, since the rôle of the food supply in the regulation of the *Calanus* population can only be determined when the minimum requirements for metabolism and growth are known. The discrepancy between the requirement calculated from oxygen consumption measurements and that estimated from measurements of the filtering rate and the concentration of particulate matter in the sea has been mentioned above.

It was hoped that the experiments of Series B reported herein would resolve this difficulty, but the heavy mortality occurring in them makes definite conclusions unsafe. However, a higher percentage of copepods attained the moulting stage in Experiment B4 with a minimum concentration of diatoms, than in Experiment B2 with ten times as many, although there was one successful moult in the latter experiment against none in the former. Presumably each copepod in Experiment B4 received not over one-sixteenth of the food requirement calculated from oxygen consumption. It is unlikely that the filtering rate in B4 was sixteen times greater than in any of the experiments in which the rate was measured. Thus it is probable that the minimum nutritive requirement for growth is less than previously believed,

though it is as yet impossible to set a definite figure. Why higher concentrations of food have so little effect on the rate of growth is problematical unless the concentration in B4 itself is well over the required minimum. Another problem is the reason for the rapid growth of *Calanus* in harbor water. Possibly qualitative as well as quantitative differences in the food supply are involved in this case.

When larger particles serve as food the effective filtering rate is probably higher. If so, the concept of the feeding rate taken at the beginning of this work—namely the volume of water filtered per unit of time—must be modified. Instead the feeding rate must be considered as the number of particles of a given type captured per unit of time when present in a given concentration.

It follows that the investigation of the minimum concentration of food which will support life and growth in *Calanus* and in other species must employ several types of food organisms. The problem is a complex one, for it is possible that the metabolic rate of *Calanus* varies with the amount of food taken in as well as with other environmental factors.

Summary

(1) The filtering rate of *Calanus finmarchicus* on the diatom, *Nitzschia closterium*, was measured under different conditions of diatom concentration, light and temperature, by observing the reduction in concentration of the diatoms. Change in diatom concentration had no effect on the rate of filtration. There was some evidence of a diurnal feeding rhythm with greater activity at night. Feeding was most rapid at 8° C., slower at 13° and 3°.

(2) Studies on the growth of *Calanus* supplied with different concentrations of diatoms indicated that the food requirement is less than has been calculated from oxygen consumption measurements. This is also borne out by comparing the amount of particulate organic matter in the sea with the rate of feeding measured in the laboratory.

(3) The view that the feeding activity of *Calanus* is automatic filtering is favored by these experiments.

(4) Approximately one-half the organic nitrogen content of *Nitzschia* is in a form available to *Calanus*.

(5) These results are correlated with the ecology of *Calanus* as studied in the field.

The author is indebted to Dr. George L. Clarke for many helpful suggestions, and to Miss Margaret Frothingham for technical assistance.

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CYST FORMATION IN THE GLOMERULAR TUFTS OF CERTAIN FISH KIDNEYS

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In an earlier study (Grafflin, 1933), the paradox, in an old specimen of daddy sculpin (*Myoxocephalus scorpius*), of a kidney showing many glomeruli anatomically but no glomerular function physiologically was satisfactorily explained. The glomeruli had been rendered incompetent by degenerative changes affecting both the vascular tufts and the neck segments, so that it was practically impossible to find a single glomerulus which on anatomical grounds could be considered functional. In young fish of the same species there was found adequate anatomical basis for the varying, but low, glomerular function which could be demonstrated physiologically. However, considerable degeneration was already present in the youngest specimens examined, and these changes became steadily more prominent with increasing age (as judged by weight). For a complete discussion of the glomerular changes noted, the original paper should be consulted. Some glomeruli, relatively quite infrequent, exhibited cystic cavities in their vascular tufts, and this is the particular problem which concerns us here.

> "Probably the most interesting glomeruli in this material are those showing what may be called a central cystic degeneration. In some instances the tuft shows a more or less spherical and very well-delimited cavity which is either entirely free from coagulum or shows it in only small amounts. These clear spaces show a wide variation in size. . . . In other tufts the cyst shows rather poor delimitation and considerable amounts of coagulum." (Grafflin, 1933, p. 65.)

At that time such cyst formation had not been observed in the glomeruli of any other species (fish or higher vertebrates). Although the specimens of *M. scorpius* showed rather numerous parasites in the kidney, all of the evidence indicated quite clearly that the parasitism played no rôle whatsoever in the observed glomerular changes. Under the circumstances, one would reasonably be led to the conclusion that the cysts represented one manifestation of the generalized process of glomerular degeneration. However, the following statement was made: "The

[1] Fellow of the John Simon Guggenheim Memorial Foundation (1934). The specimens of *Ophichthys, Crenilabrus* and *Corvina* were collected at the Stazione Zoologica, Naples, Italy. I wish to thank Professor R. Dohrn for his many kindnesses while I was a guest in his laboratory.

sharp delimitation of these central cysts in some cases suggests that we may even be dealing here with a malformation of the tuft" (p. 66). In the course of the last several years instances of cyst formation have been found in the glomerular tufts of seven additional species, two of them lungfishes, four of them marine teleosts, and one of them an arid-living reptile (the horned toad). None of these species exhibits the generalized glomerular degeneration characteristic of *M. scorpius*. Further discussion of the problem will be deferred until after a description of the findings.

Lungfishes

Protopterus æthiopicus (*African lungfish*)

In one of the available specimens (No. 21) two striking instances of cyst formation were observed, of widely different sizes and both exhibiting a delicate coagulum (Figs. 1 and 2). In another specimen (No. 34) two cysts were likewise observed (Fig. 3). Though closely adjacent, they are in different glomerular tufts, which are located, however, in the same glomerular cluster. In these latter instances the cyst contains much heavier coagulum, including what appears to be cellular debris, and in addition some well-formed cellular elements, presumably inwandering leucocytes. All four of these cysts are sharply delimited, and are lined by a flattened, endothelium-like epithelium (particularly well shown in Fig. 3). Immediately outside of this epithelial lining there is a well-defined basement membrane, which stands out quite clearly in Fig. 3. In comparison with non-cystic glomeruli, there is no increase in cellularity of the tuft, and no detectable abnormality of

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Explanation for Plate I

Figs. 1 and 2. A large and a small cyst in glomerular tufts of *Protopterus æthiopicus*. Iron hematoxylin and orange G.

Fig. 3. Two cysts, side by side, in adjacent glomerular tufts of *Protopterus æthiopicus*. Heidenhain-azan. × 410.

Fig. 4. Parasite in glomerular tuft of *Protopterus æthiopicus*. Iron hematoxylin and orange G.

Fig. 5. Small cyst in glomerular tuft of *Lepidosiren paradoxa*. Iron hematoxylin and orange G.

Figs. 6, 7 and 8. Cysts in glomerular tufts of *Myoxocephalus octodecimspinosus*. Heidenhain-azan.

Fig. 9. Glomerular tuft of *Myoxocephalus octodecimspinosus*, showing extensive region of degenerative change, interpreted as probable precursor of cyst formation. Heidenhain-azan.

All sections 5 μ. All microphotographs at × 350 except Fig. 3. All cysts, except the small one to the left of Fig. 3, photographed at point of greatest cross-sectional area.

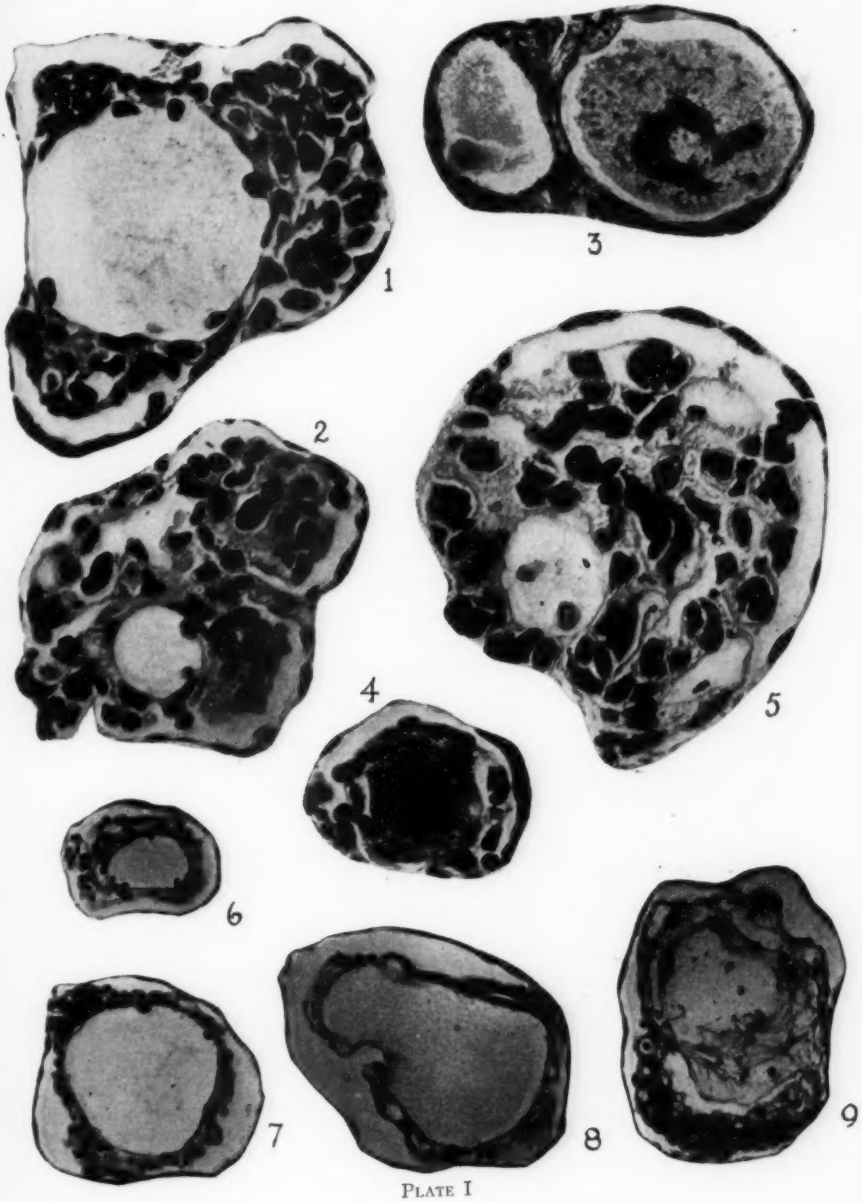

PLATE I

the afferent and efferent vessels or of the peripheral capillaries. The intracapsular spaces are free from coagulum, the ciliated neck segments are normal, and the first portions of the proximal convoluted segments are entirely comparable with those of adjacent nephrons showing no glomerular abnormality. In Specimen No. 21 the presence of a parasite in the glomerular tuft is a not infrequent finding. These parasites always exhibit a characteristic structure, which is shown in Fig. 4. Careful study of the material has failed to unearth any positive evidence that the parasites may ultimately be associated with the appearance of cysts such as those described above, but for the present such an association cannot be denied and must be left an open question.[2]

Lepidosiren paradoxa (*South American lungfish*)

In one of the specimens available a single small cyst was observed (Fig. 5). It is essentially similar in structure to the cyst shown in Fig. 2. It differs in having a less rigidly spherical outline and in containing, in addition to coagulum, a few formed cellular elements. The general statements made above concerning the cystic glomeruli of *Protopterus* likewise apply to this instance in *Lepidosiren*. Although parasitic remains are present in the kidney, they have never been observed in the glomeruli, and it is concluded that they have nothing to do with the formation of the cyst in question.

EXPLANATION FOR PLATE II

FIGS. 10, 11, 12, 13 and 14. Five instances of cyst formation in glomerular tufts of *Ophichthys imberbis*. 5 μ.

FIG. 15. Normal glomerular tuft of *Ophichthys imberbis*, without cyst formation, showing large intracapsular space frequently observed in the present material. 5 μ.

FIGS. 16 and 17. Isolated instances of cyst formation in glomerular tufts of *Crenilabrus pavo* (Fig. 16) and *Corvina nigra* (Fig. 17). 5 μ.

FIGS. 18 and 19. Two instances of cyst formation in glomerular tufts of the horned toad, *Phrynosoma cornutum*. 10 μ.

All sections stained with hematoxylin and eosin; all microphotographs at × 350; all cysts photographed at point of greatest cross-sectional area.

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[2] These specimens were collected in Africa by Professor Homer W. Smith. For the sake of completeness the following data are given. Specimen No. 21: collected in July, 1928; kept alive in dry estivation from November 1, 1928, for 427 days; replaced in water for ten days and killed, as it seemed about to die after the appearance of superficial infection. Specimen No. 34: collected in July, 1928; kept in water until January 10, 1930, being fed regularly; accidentally killed by exposure to cold. From a study of the available material, there is no evidence that a period of estivation has any bearing whatsoever upon the formation of cysts.

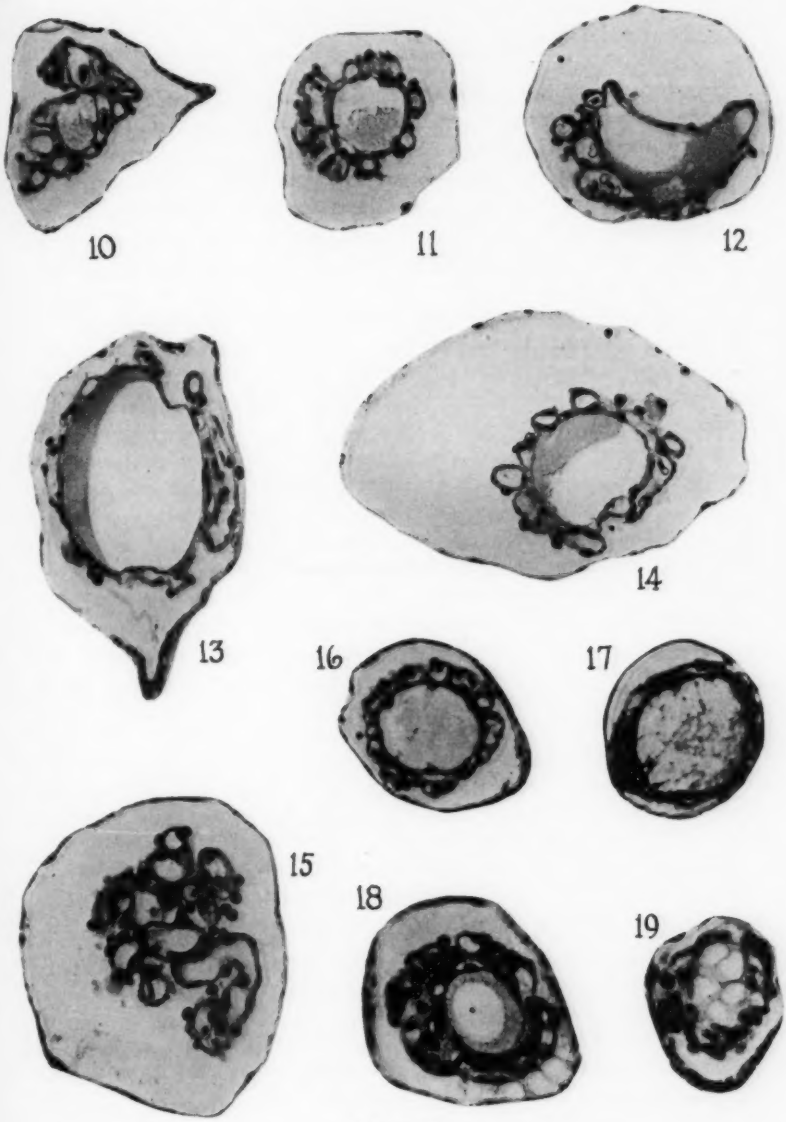

PLATE II

MARINE TELEOSTS

Myoxocephalus octodecimspinosus (sculpin)

As reported in detail elsewhere (Grafflin, 1937), the glomeruli of this species exhibit a wide variation in size and vascularity, but are in no sense the seat of generalized degenerative processes such as characterize the glomeruli of the closely related daddy sculpin (*M. scorpius*). Nevertheless, glomerular cysts have been found to occur in all of the six specimens carefully examined. In five of these specimens such cysts are present in only relatively small numbers. In the sixth, in one restricted portion of the kidney, cystic glomeruli comprise almost one-third of the total glomeruli. No reason for their large number in this region is apparent. Three examples of cystic glomeruli, to show the range in size encountered, are given in Figs. 6, 7 and 8. The cysts are sharply delimited, are lined by a markedly flattened epithelium (the nuclei of which are particularly apparent in Fig. 6), and contain coagulum of varying density. As in the lungfishes, the intracapsular spaces are free from coagulum, and the afferent and efferent vessels and the peripheral capillaries are apparently normal, as are the associated neck and proximal convoluted segments. A careful search for some lead as to the mechanism by which these cysts might arise yielded only the isolated glomerulus shown in Fig. 9, which is nevertheless very striking. While the lower part of the tuft retains essentially normal glomerular organization, the bulk of the tuft has lost it completely, and exhibits for the most part a coarse, stringy meshwork containing numerous naked nuclei. It seems reasonable to suppose that the degenerative process, which is here so clearly apparent, might eventually result in the formation of a large cyst of the type shown, for example, in Fig. 8. Small unicellular parasites regularly occur in the sculpin kidney. However, since they are never found in the glomeruli, it is concluded that they play no rôle in the formation of the cysts.

Ophichthys imberbis

In material from the caudal kidney of the single specimen available for study (Naples—weight 28 grams), five instances of cyst formation have been observed (Figs. 10–14). In all cases the ciliated neck segment is fully patent and entirely normal in appearance, and the intracapsular space shows no trace of coagulum. That the relatively large size of the intracapsular space (see particularly Fig. 14) bears no essential relationship to the presence of the cysts is clear from an examination of the normal glomeruli of this specimen, in which this space is frequently unusually large (Fig. 15). The cysts vary in size, but are

all sharply delimited and are lined by a flattened epithelium. They contain a considerable amount of coagulum, which has withdrawn to one side of the cavity, presumably in the course of fixation. The capillaries persisting in the outer rim of the tuft are normal in appearance and fully patent, and the visceral epithelium is identical in thickness and appearance with that of the normal glomeruli. The afferent and efferent vessels are likewise normal. The cysts tend to be spherical in shape, but in one case (Fig. 12) the cyst wall has collapsed, perhaps in the course of fixation. The glomerulus shown in Fig. 12 is to be compared with one previously described in the daddy sculpin (Grafflin, 1933, Figs. 7 and 15).

In three instances (Figs. 11, 12 and 14) the ciliated neck can be readily traced into the first portion of the proximal convoluted segment, which is entirely normal in appearance. In another case (Fig. 10) the sections do not include the transition. In the fifth instance (Fig. 13) the ciliated neck, approximately 100 μ in length, passes into a curious segment with flattened cuboidal epithelium, which shows neither cilia nor brush border. The cytoplasm is scanty and lightly eosinophilic; the nuclei are closely packed but show no signs of degeneration. This segment persists for about 250 μ, at which point it shows a transition to the normal epithelium of the first portion of the proximal convoluted segment.

Crenilabrus pavo

In material from the caudal kidney of the single specimen available for study (Naples—weight 120 grams), a single instance of cyst formation was observed (Fig. 16). The cyst is spherical and sharply delimited, is lined by a flattened epithelium, and contains a moderate amount of coagulum. The peripheral capillaries and the afferent and efferent vessels are entirely normal in appearance, and the visceral epithelium is unthickened. There is a very delicate coagulum in the intracapsular space. The ciliated neck segment, which is very short, is fully patent, and the associated first portion of the proximal convoluted segment shows no abnormality.

Corvina nigra

The solitary instance of cyst formation observed in this species (single specimen, caudal kidney; Naples—575 grams) is illustrated in Fig. 17. The cyst is essentially spherical, is lined by a flattened epithelium, and exhibits a rather coarse, stringy coagulum. A few naked nuclei, fairly well preserved, are found scattered at random through the

coagulum. The peripheral capillaries, few in number, contain normal red cells, and the afferent and efferent vessels show no detectable abnormality. The glomerular membrane is in many places appreciably thickened. The neck of the tubule is patent, though the lumen is quite small, and the first portion of the proximal convoluted segment is entirely normal in appearance.[3]

Horned Toad (Phrynosoma cornutum)

In surveying the available sections of the kidney of the horned toad, which is an arid-living reptile, two instances of cyst formation, entirely comparable with those observed in the fishes, were found (Figs. 18 and 19). Only a portion of the glomerulus shown in Fig. 18 is present in the sections, and both the afferent and efferent vessels and the neck segment are absent. The cyst is spherical and well-delimited, is lined by a flattened epithelium, and contains a considerable amount of coagulum. In the two sections adjacent to the one photographed there is present a dense, irregular, deeply basophilic mass of debris, which occupies perhaps one-quarter of the total cross-sectional area of the cyst. The peripheral capillary loops contain normal red cells, and show no thickening of the glomerular membrane as compared with the normal. There is a distinct coagulum in the intracapsular space. This glomerulus has formerly been briefly noted by Vilter (1935, p. 383). The appearance of the cyst in Fig. 19 is deceptive, due to the manner in which the coagulum has been precipitated. Actually the cyst is sharply delimited from the surrounding tissue, and is lined by flattened epithelium. The glomerular membrane is not thickened, and the peripheral capillary loops contain normal blood cells. The afferent and efferent vessels seem entirely normal, and the neck segment is fully patent.

Discussion

In seeking for an explanation for the formation of the cysts described above, we are led to the following considerations:

(1) All of the evidence indicates quite clearly that the presence of parasites in the kidney has no bearing upon the formation of cysts, except in the case of one specimen of *Protopterus* (No. 21). In this specimen, the tendency of the parasites to locate in the glomerular tuft is suggestive. The parasite might become walled off, and, with the sub-

[3] In this specimen one small, degenerate avascular tuft was observed which showed a central cavity containing basophilic debris. The peripheral rim of tissue was hyalinized and almost structureless, still containing scattered nuclei and nuclear fragments. This cavity is in no sense typical of the cysts discussed here, and will not be further considered.

sequent evacuation, or degeneration and absorption, of the organism, cystic cavities of the type observed might persist. However, there is no direct evidence in the material at hand that such is the case. From the available evidence, it is concluded that the glomerular cysts are formed predominantly, or entirely, on some basis other than parasitism of the glomerular tuft. If parasitism can play a causative rôle, it is a completely separate process and of minor importance for the present problem.

(2) It seems almost certain that at least some of the cysts are formed as the result of a degenerative process in the glomerular tuft. In favor of this view are some of the pictures observed in the kidney of *Myoxocephalus scorpius* (Grafflin, 1933) and the striking glomerulus observed in *Myoxocephalus octodecimspinosus* (Fig. 9).

The two instances of cyst formation in glomerular tufts of the horned toad are particularly interesting. In the first place, this is the only species above the fishes in which such cysts have yet been recorded. In the second place, the reptilian glomerulus usually exhibits a central, avascular, cellular core, which, according to Regaud and Policard (1903) and Cordier (1928), is made up of connective tissue. Such a core is constantly present in the glomeruli of the horned toad (Marshall and Smith, 1930; Vilter, 1935). In the two glomeruli illustrated in Figs. 18 and 19, the cystic cavities occupy the region of the typical central cellular core, and replace it to such an extent that no characteristic portion of the core is any longer recognizable. One is led to wonder whether the cysts might not have arisen as the result of degeneration of the central avascular area. The irregular mass described above for the larger cyst (Fig. 18) is opaque and amorphous, and has all the appearance of calcified debris; it might be construed as the remains of the original core.

Let us now examine the available fish material in the light of these considerations for the horned toad. The glomerular tufts of *Crenilabrus* tend to be somewhat cellular, and one occasionally finds a central avascular core. The tufts of *Corvina* tend to be quite cellular, and it is not infrequent to find a typical central cellular core, entirely comparable with that seen in the horned toad and pigeon (see below). In *Myoxocephalus scorpius* (Grafflin, 1933) many tufts show a markedly cellular center, which may be entirely avascular. In an earlier study (Grafflin, 1929) it was shown that the relatively few glomerular structures present in the kidney of the adult goosefish (*Lophius piscatorius*) have lost all connection with renal tubules. The important fact for the present problem is that eight out of thirty-one of these

"pseudoglomeruli" which were carefully studied showed a central degeneration of the glomerular tuft. In seven of them the center of the tuft was hyaline, eosinophilic and entirely avascular, and showed a few scattered nuclei and nuclear fragments; in two of these seven this central area was vacuolated in addition.[4] In all of these tufts the central hyaline area was very sharply delimited from the peripheral tissue.

If our well-delimited cysts are to be interpreted in terms of degeneration and liquefaction of a central avascular portion of the tuft, certainly it is just as conceivable that such a process could occur in all of the fishes described above as in the horned toad. In favor of this interpretation are the amorphous mass in one of the cysts of the horned toad and the scattered nuclei in the cyst of *Corvina*.

However, ranged against such an interpretation are the following facts. (1) The two cysts observed in the horned toad are isolated instances, whereas one might reasonably expect them to be numerous on this basis. (2) In the pigeon, whose glomeruli likewise exhibit a cellular avascular core, an extensive search of abundant material failed to reveal a single instance of cyst formation (Vilter, 1935). (3) Though many glomeruli of the goosefish show marked degeneration of the central part of the tuft, no instance of cyst formation in such a tuft has yet been observed. (4) In the specimens of *Ophichthys, Lepidosiren* and *Protopterus,* the glomerular tufts are well vascularized, and no accumulations of cells have been observed which in any way suggest a cellular avascular core. Similarly in the sculpin (*M. octodecimspinosus*), in the usual range of glomerular size, no well-defined central core has yet been observed. (5) Some of the cysts are very small, and it seems perfectly clear that as we see them they are at their maximum size. It is hardly conceivable, in view of the findings in the pigeon, that an avascular region of the size represented by these small cysts would undergo degeneration. (6) The cysts are in general spherical, and give every indication of having contained fluid under pressure. If we were dealing merely with a degeneration of the central portion of the tuft, one would more logically expect collapse of the tuft rather than distension of the type observed.

In summary, while some of the glomerular cysts are apparently formed on the basis of a degenerative process in the glomerular tuft,

[4] The eighth tuft was very small and atrophic, and showed a central degeneration to the point of cavity formation, the cavity containing granular debris. The picture is not at all typical of the cysts discussed here, and will not be further considered.

In the legend to Fig. 7 of this earlier paper there is an obvious error. It is clear from the illustration that no ciliated neck segment is present, and that the intracapsular space opens directly into a segment whose cells exhibit the brush border characteristic of the epithelium of the proximal convoluted segment.

it seems unlikely that all of the cysts which we have observed could arise in this manner.

(3) It is suggested that many of the glomerular cysts herein described probably result from a malformation of the glomerular tuft. The cyst might be laid down early in embryological development, or it might be formed rather late, when glomerular organization is already well advanced. In the latter case two possibilities immediately suggest themselves: (*a*) the walling-off of a deep crevice between adjacent lobulations of the tuft; (*b*) the occlusion at both ends of a portion of one of the glomerular capillaries. On either basis one could readily understand (1) the flattened epithelial lining of the cyst; (2) the subsequent enlargement of the cyst without, at the same time, any encroachment upon or collapse of the surrounding capillaries; and (3) the presence within the cyst of coagulum, which would represent simply a seepage of plasma proteins into the completely closed cavity. Also, such a mode of formation would be consistent with the small size of some of the cysts and the failure to find, in adult animals, more than occasional suggestive intermediate stages in cyst formation.

Summary

Well-delimited glomerular cysts have been observed in the kidneys of the following species of fishes: *Protopterus æthiopicus, Lepidosiren paradoxa, Myoxocephalus scorpius, Myoxocephalus octodecimspinosus, Ophichthys imberbis, Crenilabrus pavo, Corvina nigra;* and in the horned toad, *Phrynosoma cornutum.* It is concluded that these cysts are probably formed in two ways: (1) on the basis of a degenerative process in the glomerular tuft; (2) as the result of a malformation of the glomerulus in embryological development.

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